Vol. XL No. 7

July, 1951

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

Proof of Safety: Some Interpretations 305
A. J. Lehman

Modified Glassware and Techniques Designed to Increase the Precision of the U. S. P. XIV Vitamin A Assay 309
James A. Brown

A Study of Folic Acid Stability in Solutions of the B Complex Vitamins 313
Alfred R. Biamonte and *George H. Schneller*

Antifungal Activity of a Series of Thiocyanates 321
L. Landis, D. Kley, and *N. Ercoli*

The Fungistatic Properties of Acenaphthene Derivatives 325
James E. McDavid and *Troy C. Daniels*

Studies on Sex Difference in Resistance to Ouabain in the Albino Rat 327
Harald G. O. Holck and *Kazuo K. Kimura*

Some Pharmacologic Actions of 53 Derivatives of Thiazole with Substituents in the 2- and 4-Positions 333
Bradford N. Craver, Walter Barrett, Anne Cameron, and *Jean Feickert*

Investigations of Di(acetylhydroxyphenyl)isatin. I. Quantitative Excretion in Rats 340
R. G. Sanders, H. M. Baum, and *G. J. Straub*

A Procedure for the Isotopic Synthesis of *Alpha*-Amino Acids with Reference to Isoleucine 341
George B. Ceresia, G. L. Jenkins, and *Ed. F. Degering*

A Method for Biologic Standardization of *Veratrum viride* Based on the Survival Time of *Daphnia magna* 345
R. O. Craw and *Alan E. Treloar*

The Occurrence of a Diphenyl Isatin in California Prunes 348
H. M. Baum, R. G. Sanders, and *G. J. Straub*

Contributions to the Mode of Action of Sympathetic Blocking Agents 350
Theodore O. King

Determination of *Gamma*-1,2,3,4,5,6-Hexachlorocyclohexane in Lotions and Ointments 357
John H. Fossum and *John A. Riddick*

Assay of Pharmaceutical Tablets Containing Calcium by the Schwarzenbach Method 359
George B. Griffenhagen, Joan L. Pfisterer, and *Selma K. Sloth*

Book Reviews 361

Advertising ..... Cover pages 2, 3, 4, and pp. I–IV

Copyright, 1951, by the American Pharmaceutical Association

Basic Approach to a RISING Sales Curve

THE SUREST ROUTE to *lasting* sales-improvement is product-improvement ...and often the most effective way to improve a product is through *improved* flavor or scent!

As one of the world's great suppliers of Essential Oils, Perfume Oils and Concentrated Flavors, MM&R has cooperated in the establishment of individualized odor or flavor personalities for *hundreds* of leading products! Perhaps MM&R can now be of important service to *you!* More than 56 years of specialized experience are at your command, without obligation.

For full information, write to the MM&R Technical Service Dept. today.

Builders of
PRODUCT PREFERENCE
through
QUALITY ESSENTIAL OILS
PERFUME OILS • FLAVORS
ODOR NEUTRALIZERS
BALSAMS • OLEORESINS • COLORS
Since 1895

MAGNUS, MABEE & REYNARD, INC.

SINCE 1895 ... ONE OF THE WORLD'S GREATEST SUPPLIERS OF ESSENTIAL OILS

16 DESBROSSES STREET, NEW YORK 13, N.Y. • 221 NORTH LASALLE STREET, CHICAGO 1, ILL.

San Francisco: Braun-Knecht-Heimann Co. • Los Angeles: Braun Corp. • Seattle, Portland, Spokane: Van Waters & Rogers, Inc.

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

SCIENTIFIC EDITION

Committee on Publications

George D. Beal, *Chairman*
B. V. Christensen
Robert P. Fischelis
Don E. Francke
Hugo H. Schaefer

Editorial Advisory Board

L. W. Busse
John E. Christian
T. C. Daniels
L. W. Hazleton
E. J. Hughes
H. W. Youngken, Jr.

JUSTIN L. POWERS, EDITOR

VIVIAN OVIATT, EDITORIAL ASSISTANT

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 250 words including a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL center headings should be avoided so far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "*a*" and continuing in natural order.

Published by the American Pharmaceutical Association under supervision of the Committee on Publications with the approval of the Council. Publication office, 20th and Northampton Sts., Easton, Pa. Editorial office: Scientific Edition, 2215 Constitution Ave., Washington 7, D. C.

Annual Subscription—Journal of the American Pharmaceutical Association, complete (both editions): United States and Pan America, $7.00; Canada, $7.70; other foreign, $8.00; members of the American Pharmaceutical Association with dues, $4.00. Each edition, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50. Single numbers, either edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

Claims for missing numbers will not be allowed (1) if received more than 60 days from date of issue, (2) if loss was due to failure to give 30 days notice of change of address, or (3) if reason for claim is "missing from files."

Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the fifth; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

Literature citations should be grouped at the end of the manuscript under the heading "References." The citations should be numbered consecutively with full sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by *Chemical Abstracts* in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, 79, 289(1927).

References to books should be in the order given, (1) author's surname and initials, (2) title of the book, (3) edition, (4) name of publisher, (5) place of publication, (6) year of publication, (7) volume, and (8) page number. A complete book reference may be illustrated as follows:

(1) Gilman, H., "Organic Chemistry," 2nd ed., John Wiley and Sons, New York, 1943, Vol. 2, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e.g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mm. cm., and L.

Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

Numbers and measurements, etc., which are to appear on the axes of the drawing should be indicated in pencil outside the axes, in order that they may be set in type. Captions and legends should be placed *below* the diagram (not on it), or, if lengthy, collected on a separate typewritten list, identifying each by its proper figure number and including whatever acknowledgments are necessary.

Photographs should never be rolled, and paper clips should not be used unless guarded by a piece of paper folded several times to prevent an indentation in the photograph. All drawings and photographs should be protected from bending and from the chance of having their edges torn while in transit. Do not write on the back of a photograph. Any indentation in the photograph, however slight, will be likely to show as a blemish in the reproduction. All figures submitted must be referred to in the text of the manuscript, and should be numbered consecutively with Arabic numerals, e.g., Fig. 1, Fig. 2, etc. Kymograph tracings should not be submitted with the original experimental legends which are likely to have been hastily or carelessly inscribed. Authors may find it worth while to employ a professional draftsman to make the drawings in proper form for reproduction.

Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretive statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript and the author should indicate as nearly as possible the positions in which the tables should be set. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

LIST OF MISCELLANEOUS REPRINTS

Interesting and Instructive Pharmaceutical Articles—*(15¢ each).*

Action of Drugs on Parasympathetic Nervous System, by M. W. Green
A. Ph. A. Official Roster and Pharmaceutical Directory
Are Animal Experiments Needed? by A. C. Ivy and A. F. Zobel
Clinical Toxicity of Thiouracil
Suggested Plans for Hospital Pharmacies
Practical Guides to New F.D.A. Labeling Regulation
Proposed Uniform State Pharmacy Act
The Relation of the Pharmacopoeia to the Pharmaceutical Profession
Some Modified Formulas for Dermatotherapy
Survey of Pharmacy Laws, by P. A. Costello

Pictures—*($1 each; $1.50 any 2)*

Frederick B. Power
Pharmakeutike—Sculptural Panel
Phos-Kai-Elpis—Sculptural Pane
The Laboratory (*a famous misnamed picture*)

Miscellaneous Items

A. Ph. A. Code of Ethics—In two colors, sizes 12 x 18″ or 10 x 13½″, 25¢
Badianus Manuscript—color plate reprint, 50¢
The Oath and Prayer of Maimonides (2 colors), 25¢
Table of Metric Doses, 1½¢ each, 100 for $1.25

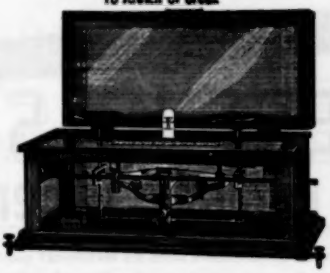
Scale illustrated is our
No. 540 all Glass Model

Capacity—4 oz. (120 grams)
Sensitivity—1/10 grain
Finish—Genuine light mahogany
with chrome plated parts
Price—$135.00 (subject to
change without notice)

PROMPT DELIVERY
on this as well as other class A scales

No. W35 (all glass in lid).....$100.00
No. W33.....90.00
All With Stainless Steel Pans

Our scales have no bands or wires to stretch or break



High quality
Prescription
Scale made by
HENRY TROEMNER
Manufacturer of the Finest Scales for the Druggist
Since 1840
911 Arch Street
Philadelphia 7, Pa.
(Send for Circular A-A)

COLLEGE OF PHARMACY AND CHEMISTRY

A co-educational institution offering degree courses in Pharmacy (B.S. Pharm.) and Chemistry (B.S. Chem.). Graduation from an accredited high school or the equivalent is required for admission.

Because the College is located in the pharmaceutical manufacturing center, ample opportunities are open to the students to become acquainted with the most modern and practical methods. The College, the Alumni, and Detroit pharmacists all cooperate to assist students in securing part-time employment. Write for catalog.

DETROIT INSTITUTE OF TECHNOLOGY
2020 Witherell Detroit 26 Michigan
303 Downtown YMCA • Grand Circus Park

The Massachusetts College of Pharmacy
Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the field of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in a great medical and educational center. It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy. A few fellowships are available.

For additional information, write to HOWARD C. NEWTON, Dean.

A Career in Pharmacy and the Allied Sciences
Philadelphia College of Pharmacy and Science.

Pharmacy, Chemistry, Bacteriology and Biology offer opportunities for interesting and successful careers to young men and women. B.Sc. degree courses. Graduate study leading to M.Sc., and D.Sc. degrees.
Write for catalog to
43rd St., Woodland and Kingsessing Aves., Philadelphia 4, Pa. Founded in 1821.

PHARMACEUTICAL CHEMIST

To direct product development group. Location: New Jersey. Pharmacy training and strong chemical background required for fundamental as well as practical approach to problems of stability, compatibility, etc. Ph.D. or equivalent experience. Please advise. Box 651, Scientific Edition, Journal of the American Pharmaceutical Association, 2215 Constitution Avenue, N.W., Washington 7, D. C.

CHOLESTEROL IN ITS MOST ACTIVE AND EFFICIENT FORM

the

AMERCHOLS

are naturally superior

The AMERCHOLS are surfactants which are NATURALLY good for the skin and hair. They are odorless, color free forms of cholesterol and related rare sterols which we isolate in purified form from animal tissues. They are stable in the presence of acids, alkalies and most drugs.

WE KNOW OF NO CASE OF ALLERGY DUE TO AN AMERCHOL

PHARMACEUTICAL MANUFACTURERS AND CHEMISTS:
Our research laboratories are available to you for advice and information. Our booklet will help you formulate the AMERCHOLS in O/W and W/O emulsions.

AMERICAN CHOLESTEROL PRODUCTS INCORPORATED

MILLTOWN · · · NEW JERSEY

for Aluminum
Hydroxide Gels of
uniform excellence,
remember that name...

REHEIS

Reheis FINE CHEMICALS

REHEIS COMPANY INC. · BERKELEY HEIGHTS, NEW JERSEY

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XL | *JULY, 1951* | NUMBER 7 | CONSECUTIVE NO. 14

Proof of Safety: Some Interpretations*

By A. J. LEHMAN

Interpretations are presented, according to the objectives sought, of the techniques and procedures required by the Food and Drug Administration to establish the safety of a chemical in the form of a food additive, a drug, or an ingredient of a cosmetic. Special emphasis is placed on proof of safety of chemicals used in foods because, in general, these require a more thorough toxicological appraisal than those used in cosmetics or as drugs.

AN EXPERIMENTAL procedure for establishing proof of safety of chemicals to which man may be exposed has been presented (1). The highlights of the detailed program may be summarized as follows:

I. Chemistry
   1. Solubility
   2. Chemical Characterization
   3. Quantitative Micro Determination
II. Acute Toxicity
   1. Oral LD_{50} in Several Species
   2. Pharmacodynamics
III. Allergic Responses
IV. Subacute and Chronic Toxicity
V. Biochemistry
VI. Pathology

Since the publication of the detailed program, some problems have arisen regarding the interpretations which may be placed upon certain phases of the procedures. The terms listed above may be interpreted in different ways depending somewhat on the objective sought by the investigator. However, when proof of safety must be established for a large segment of the population, the interpretations which will be presented here may be somewhat different from the generally accepted idea conveyed by the terms, especially to those not familiar with toxicity tests, or to those thinking of conditions of about thirty years ago.

Man may be exposed to chemicals in the form of a food additive, a drug, or as an ingredient of cosmetics. Substances which become a constituent of the daily food require a a more thorough toxicological appraisal than those which fall into the other two categories, hence in the discussion to follow, emphasis will be placed on proof of safety of chemicals which may be directly or indirectly incorporated into the daily food supply.

I. CHEMISTRY

Solubility.—The particular interest is in the solubility of the chemical in materials with which it may come into contact under actual conditions of use. In the case of a plasticizer in a film proposed as a wrap for fatty foods, it is more important to know its extractability in

* Received May 11, 1951, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

lard at 30° C. during a thirty-day contact than the solubility of the pure plasticizer in an imposing list of chemical solvents under standard conditions. Further, the scope of the solubility studies depends upon the manner of use of the chemical. If the film will come into contact with many types of food, then the solvent effect of such food-type materials as pickle brine, sugar solutions, meat juice, weak sodium bicarbonate solutions, vinegar, milk, and lactic acid solutions should be known. Solubility may also have a bearing on the comprehensiveness of the pharmacological investigations which may be necessary to establish proof of safety. For example, a chemical having a solubility of the order of one part per million in the various solvents would ordinarily present less of a pharmacological problem than if the solubility were of the order of 10 or more parts per million.

Chemical Characterization.—In addition to the type of information usually developed for a chemical, i. e., its empirical and structural formula, stability, and reactivity to various reagents, etc., it is necessary to know its stability or possible reaction with foodstuffs, drugs, and cosmetics. A chemical may show certain characteristics with common laboratory reagents, but could behave differently when added to a lard which is used for deep fat frying. Again, boric acid and methenamine are relatively inert materials when placed on the skin separately, but in combination have been known to produce dermal injury by the liberation of formaldehyde. The production of an off-flavor in fruits and vegetables treated with certain insecticides is still another example of the necessity for exploring chemical reactivity beyond the more or less standard procedures for developing such information.

Quantitative Micro Detection.—Here emphasis is placed on the detection of a chemical in the presence of interfering substances which may be found in drugs, cosmetics, and especially foods. In dealing with this problem, a micro method is one which should detect one part per million or less in the presence of milk, acetylsalicylic acid, wine, or any other substance offered for human consumption to which the chemical tested may be added. Furthermore, the method must be specific. A system of analysis based on organic chloride determinations for the detection of tissue distribution is of little or no value in tracing the fate of the chlorinated insecticides.

II. ACUTE TOXICITY

Oral LD_{50}'s.—The first item under acute toxicity calls for oral LD_{50}'s in several species of animals and should include rodents and nonrodents. Considerable emphasis is placed on the use of several species, because if a wide species difference is evident an almost immediate unfavorable atmosphere begins to surround the future usefulness of the chemical under test. Man is heterogeneous, and from past experience animals quite faithfully predict what may be expected in humans. Furthermore, an adverse response in one individual in a hundred is, to our way of thinking, a 100 per cent response in this individual rather than a 1.0 per cent response in a hundred individuals.

In interpreting the results of acute toxicity tests we have generally maintained that if a substance shows an oral LD_{50} of less than 1 Gm./Kg. of body weight in any species of animals, it should at least tentatively be classed as a poisonous or deleterious substance, and as such excluded by law from all foods except where its use is required or cannot be avoided. This value does not apply to drugs where therapeutic efficacy is balanced against toxicity.

Pharmacodynamics.—It is almost axiomatic that if a chemical has significant pharmacodynamic effects, its use as a food additive or as a cosmetic ingredient would be severely limited. Obviously, in the case of drugs, pharmacodynamic action is indispensable.

III. ALLERGIC RESPONSES

Where sensitization tests on guinea pigs turn out negative it is frequently desirable to verify the results on human beings. Here again repeated exposures are a crucial feature of the test. Patches containing the test material are applied to the skin every other day, or three times weekly until a total of 10 applications have been made. Two weeks after the tenth patch a "challenge" patch is applied and the response to it shows whether or not the subject has become sensitive. In tests of this type at least 200 subjects should be used. Absence of reaction in this number does not, of course, insure against annoying or even dangerous allergic incidents.

IV. SUBACUTE AND CHRONIC TOXICITY

This aspect of the program has not been subject to any serious misinterpretations. Occasionally the usefulness of a ninety-day sub-

acute experiment on dogs and rats has been questioned. Chronic toxicity studies are the most time-consuming as well as the most costly of the entire program, and it is only reasonable to run a pilot experiment first as a guide for establishing the dosage levels to be fed in the long-term experiments. Furthermore, dosage levels in a pilot experiment can be pushed upward to the limit of tolerance. This is for the purpose of determining the nature of the toxic effect, if any, and demonstrating what tissues or organs bear the brunt of the toxic insults. It is possible that animals on the higher levels may succumb before the end of the ninety-day period. This actually may be one of the objectives of the experiment and gives much valuable information on the nature of the injury which caused death. On the other hand, the loss of animals on the higher levels in a life time study on rats is serious because one of the objectives of a long-term feeding experiment is to expose animals from the weanling stage through senility to as high a level of the chemical as can be tolerated and still have survivors at the end of the two-year observation period. Thus, if all animals on certain high levels succumb by the end of the first year of feeding, it may be necessary to place additional animals on new dosage levels with the consequent loss of one year's time to complete the program.

Sometimes the results of the ninety-day feeding test may in themselves be sufficient to classify the compound as a poisonous or deleterious substance. When this happens it is well to pause and carefully reconsider the question of necessity for use before proceeding to the chronic toxicity tests. It can be very annoying to discover that one has accumulated all the scientific data needed for setting a tolerance, but on a compound whose use is not required or can be avoided by good manufacturing practice.

It may be of interest to mention a criticism which is occasionally leveled against the necessity of lifetime studies. To illustrate, suppose the potential use of a chemical (I) may be so promising that production could reach 100 million pounds a year. The economics of this item are such that the cost of the research to appraise toxicity is easily justified. At the same time another chemical (II) manufactured by the company may reach a production of only 100,000 pounds a year. This chemical, although used in lesser amounts but more toxic, may have a distribution as wide as that of I. The question is asked: Why should it be necessary to spend a like amount of money in appraising the toxicity of chemical II as for chemical I when dollar sales value of II may be only one-thousandth that of I. A little sober reflection will reveal that from the health hazard standpoint the impact upon the consumer would be about equal for the two chemicals; hence pharmacological studies must be as comprehensive for II as for I.

The question most often asked is: Why the necessity for carrying on experiments beyond one year? Will not all aspects of chronic toxicity be demonstrated within twelve months? The answer is an emphatic *NO*. Experiments in these laboratories have demonstrated many serious effects in the last quarter or third of a twenty-four-month feeding experiment which would not have been observed if the experiments had been terminated earlier. Examples follow.

(*a*) Bladder stones and enlargement of testes in rats fed certain of the glycols.

(*b*) Ear tumors in rats chronically poisoned with ergot.

(*c*) Vitamin E deficiency in rats fed rancid lard.

(*d*) Liver tumors in rats in chronic selenium poisoning.

(*e*) Thyroid malfunction in rats fed "P-4000" sweetening agent.

(*f*) Enlargement of the spleen and liver tumors in rats fed dulcin, an artificial sweetening agent.

(*g*) Liver tumors in rats fed thiourea.

Further justification of long-term studies is supported by the fact that the human population has people of all ages. There is substantial evidence that the human body is more easily injured at some ages than at others. In a lifetime experiment a chemical is given to animals through all age periods. Such a procedure may produce more toxic effects at one age than at another. This fact has been observed many times. A lifetime experiment is a compromise, for it is obvious that it would be a better procedure to start additional groups of animals on the experimental regimen at different ages.

V. BIOCHEMISTRY

Biochemical Studies.—In biochemical studies emphasis is placed on absorption, fate, distribution, and excretion. Considerable suspicion is cast upon any chemical which has a tendency to accumulate in the tissues. It is

recognized that cumulation may be a desirable property and actually may be an advantage in the therapeutic efficiency of certain drugs. However, for the most part tissue build-up of a toxicant is undesirable. An excellent example of how cumulation has served to make a fine discrimination of toxicity between compounds is the tissue accumulation of mercury when administered as a mercuri-phenyl and as a mercuric salt. Phenylmercuric acetate causes from 10 to 20 times as much mercury storage in the liver and kidney tissue as mercuric acetate. The phenyl compound is also chronically more toxic than the mercuric acetate. This evidence has serious ramifications into the food field where phenylmercuric acetate is the basis of certain fungicidal sprays for growing crops, and may have serious implications in therapeutics wherever phenylmercuric acetate is used.

In addition to the four points listed, two other biochemical aspects which appear to be playing a more prominent role in establishing proof of safety are the effect of chemicals on enzymes and the influence of the chemical on the metabolism of normal constituents of food.

VI. PATHOLOGY

The sixth and last major phase of the program has been thoroughly discussed (1). About all that needs emphasis here is that pathological study should be done with good control material, and against a background of experience.

FUTURE TRENDS

A few modifications which are beginning to develop for future programs of proof of safety may be mentioned. Lifetime studies may have to be extended to include other species such as the dog. Five-year feeding studies on dogs are often necessary whenever fine discriminations in toxicity must be made. More attention is being given to the influence of chemicals on some of the fundamental physiological processes of the body. It is possible that as more evidence on the effects of chemicals on enzymes becomes available, some simple screening device may be developed which will aid in reaching a decision on the future possibilities of a promising chemical. Perhaps the use of some relatively simple biological material such as the algae may be useful for the same purpose. Some experiments employing algae are now in progress in an attempt to elucidate the mechanism of the poisoning effect of certain insecticides with the very practical objective of developing specific antidotes.

Perhaps it is appropriate to mention also a phase of pharmacodynamics which undoubtedly must receive more attention than it has in the past. This has reference to adipose tissue. This tissue is not merely connective tissue which functions in a passive manner as a fat depot, but rather it is a structure possessing functions which have been likened to those of a ductless gland. Of particular interest is the high lipid content of cell membranes. Thus chemicals which have a tendency to accumulate in fat may have a far-reaching effect on cell permeability and hence on all vital processes.

REFERENCE

(1) Lehman, A. J., Laug, E. P., Woodard, G., Draize, J. H., Fitzhugh, O. G., and Nelson, A. A., *Food Drug Cosmetic Law Quarterly*, **4**, 412(1949).

Modified Glassware and Techniques Designed to Increase the Precision of the U. S. P. XIV Vitamin A Assay*

By JAMES A. BROWN†

The paper describes the construction of a glass separatory vessel which can be used also as a boiling flask. The use of this vessel in place of the conventional refluxing flask reduces the time required to perform the assay and markedly increases the possibility of good precision. Data are given as examples of the precision attainable. It also presents data to support the idea that a surprising increase in precision can result from attention to the elimination of small errors in the determination of the absorbancies of the vitamin A solutions, as opposed to the notion that small errors and the resulting lack of precision are inevitable.

THE SAPONIFICATION and extraction techniques involved in the performance of the U. S. P. assay for vitamin A (1) are time-consuming and often lead to loss of material and consequent lack of precision. The difficulties encountered in this part of the procedure are among the major obstacles to the successful performance of the assay by many analysts. Other causes of poor precision with this procedure include the errors in determining the absorbancy which, while apparently small, exert a large influence on the final result.

This paper has two purposes: first, to describe modified glassware and techniques which have reduced the time required to perform the assay and increased markedly the precision obtained in our laboratory; and secondly, to bring forth the idea that a surprising increase in precision can result from attention to the elimination of small errors in determining the absorbancies of the vitamin A solutions, as opposed to the notion that small errors and the resulting lack of precision are inevitable.

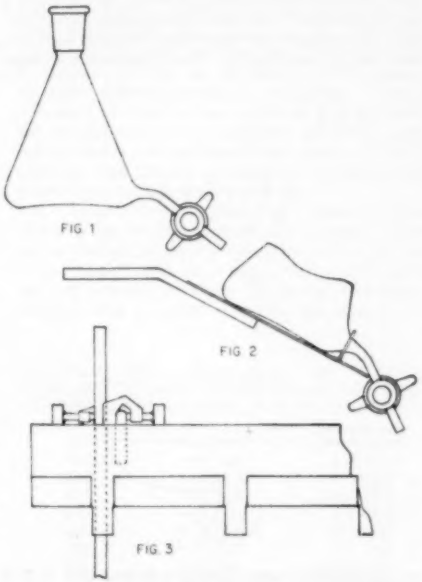


FIG. 1

FIG. 2

FIG. 3

APPARATUS

Figure 1 shows a flask that has been constructed to serve as a refluxing flask, separator, and evaporation flask. By using this modified glassware in place of the conventional glassware, the major portion of the vitamin A can be kept in a single container from the time the sample is weighed directly into it until the washed ether extract is ready to be transferred to a volumetric flask. The modified flask is made by fusing a size 2 or 3 Pyrex stopcock onto a 300-cc. Erlenmeyer flask with a 24/40 standard taper neck. (The 300-cc. size made of nonactinic glass can be obtained from the Corning Glass Works.) The point of fusion is any place on the largest circumference of the flask. The distance from the flask to the stopcock is about $1^1/_2$ in. The fusion is made so that the stopcock tube is parallel to the base of the flask, and then it is bent, midway between the stopcock and the flask, to about a 30° angle. This construction allows the contents to be removed through the stopcock without tipping the flask so far that the contents are liable to spill out at the top.

The problem of a suitable rack to hold the unconventionally shaped flask was solved very simply by making a sheet metal platform (preferably stainless steel), to which were welded two $^3/_8$-in. rods so that the whole can be supported on two ring stands or other conventional laboratory supports. Figure 2 shows a side view of the rack with flask in place, and Fig. 3 a front view. The rods were bent so that the platform is tipped to support the flasks at an angle of about 30° forward of the vertical. This

* Received January 24, 1951, from Eli Lilly and Co., Analytical Laboratories, Indianapolis, Ind.
† Acknowledgment is made to Mr. Max M. Marsh, Eli Lilly and Co. Analytical Department, for suggesting the type of rack for holding the flasks.

angle allows the phases to separate cleanly without any trapping of the lighter phase. The front of the sheet metal is turned up 1 in. back from the edge after cutting to allow for notches about $^3/_4$ in. wide for the stopcock to project through. The pieces of metal not turned up are left on, to serve as supports, to prevent the flask from tipping over forward. There are other ways, of course, of providing support for the flasks. Best results are obtained if they are supported at an angle which allows clean separation of the phases.

PROCEDURE

The sample to be analyzed is weighed directly into the flask. The specified amounts of alcohol and KOH solution are added, keeping the flask tilted backward so that the solutions and sample do not run down into the stopcock tube. The flask is then attached to the reflux condenser, still keeping it tilted backward. An electric heating mantle (250-cc. size) or an electric Kjeldahl-type heater is used as a heat source (see Fig. 4). Two Kjeldahl-type heat-

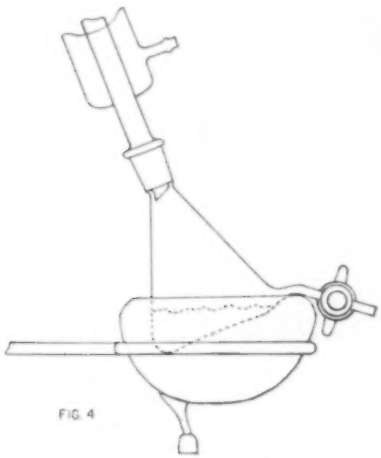


FIG. 4

ers connected in series furnish sufficient heat to boil the contents of the flask. After saponification, no harm is done if the solutions run down the stopcock tube. After cooling, the water and ether are added and the contents mixed by swirling. It is not necessary to insert a stopper, as sufficient mixing is accomplished by merely swirling the contents. After separation, the lower layer is drawn off into a conventional separator where the second extraction is made and allowed to separate. The lower layer is transferred to a second separator, and the ether layer added to the first extraction in the original flask. The third and fourth extractions are made, the fourth in the same separator as the second. After each successive ether extraction is added to the original in the original flask, the small additional amount of water phase which separates due to this addition is added to the bulk of the water phase being extracted in order to be sure all possible vitamin A is being removed from the water phase. The original flask containing all four ether extractions is mixed by swirling and the first water wash gently added while swirling. The inherent danger of an emulsion at this stage is somewhat minimized by the fact that since no stopper is used in the flask, there is less tendency to shake it too violently. With this type of flask and the swirling action during the mixings, four washes usually suffice to remove all of the alkali. After draining the last water wash completely, the flask can be placed on a hot plate, water bath, or heating mantle to evaporate part of the ether if desired. If a flat plate-type of heater is used, the stopcock is allowed to project over the edge of the plate. In this laboratory, however, superior results have been obtained when the evaporation is omitted and a 200-cc. volumetric flask is used instead of a 100-cc. flask for the ether dilution. This prevents possible oxidative losses during the evaporation and the weaker ether solution can be compensated for by proper choice of pipette and flask for the final dilution with isopropyl alcohol.

The drying of the ether extracts is accomplished without transferring to another vessel by filtering into the volumetric flask through a 30-cc. fritted glass funnel (coarse porosity) which contains a half-inch layer of granular Na_2SO_4. The Na_2SO_4 layer is prewashed with ether. It is finally washed free of vitamin A with ether, four to five washings usually sufficing to wash it clean. No trouble has been experienced either with failure of the Na_2SO_4 to remove the water or with the final ether washings failing to remove all of the vitamin A from the sulfate.

The procedure from this point follows the U. S. P. procedure and depends on the starting material, weight of sample, etc. Superior results have been obtained in this laboratory by choosing the sample weight, when possible, so that only one pipetting of the ether extracts is required. The pipetting is done directly into the selected volumetric flask, and the ether evaporated by inserting a tube and blowing nitrogen through it gently while it is rotated in a 40° water bath. In nearly all cases the sample size and dilutions can be selected so that not over 20 cc. of ether need to be evaporated in a 50-cc. or 100-cc. flask, and usually the amount to be evaporated is less than this. The last few cubic centimeters can be evaporated without heat as the U. S. P. specifies.

NOTES ON DETERMINATION OF ABSORBANCIES

The following are points which we have found need special emphasis over and above the general recommendation to use careful analytical techniques.

1. A Beckman Spectrophotometer which has been specially standardized for the vitamin A determination should be used. Rawlings and Wait (2) have pointed out many of the errors involved in the spectrophotometric determination of vitamin A. Their recommendations for obtaining instrument stability and general precision should be followed. The importance of using a stable instrument capable of exact reproducibility cannot be overemphasized. An important additional point pertinent to the U. S. P. XIV vitamin A assay in particular is that the wave-length scale of the spectrophotometer should be calibrated to correspond exactly to the

TABLE I.—EFFECT OF SMALL ERRORS IN THE ABSORBANCY READINGS ON THE FINAL RESULTS

	Correct Figure	0.001 Error at 310 mμ	0.001 Error at 325 mμ	0.001 Error at 334 mμ	0.001 Error at Each
A_{310}	0.614	0.613	0.614	0.614	0.613
A_{325}	0.722	0.722	0.721	0.722	0.721
A_{334}	0.644	0.644	0.644	0.643	0.643
$A_{corr.}$	0.6248	0.6274	0.6178	0.6291	0.6248
C	0.001014	0.001014	0.001014	0.001014	0.001014
$E_{corr.}$	616.2	618.7	609.3	620.4	616.2
% Error		0.41	1.12	0.68	**0**
When absorbancies are halved:					
A_{310}	0.307	0.306	0.307	0.307	0.306
A_{325}	0.361	0.361	0.360	0.361	0.360
A_{334}	0.322	0.322	0.322	0.321	0.321
$A_{corr.}$	0.3124	0.3150	0.3054	0.3168	0.3124
C	0.00507	0.00507	0.00507	0.00507	0.00507
$E_{corr.}$	616.2	621.3	602.4	624.9	616.2
% Error		0.83	2.24	1.41	**0**

313.16 and 334.15 mμ lines of the mercury discharge tube. Only when each laboratory does this, in addition to striving for good precision, can there be good interlaboratory agreement. The spectrophotometers as received from the manufacturer are not sufficiently accurate in this respect, because the instruments are calibrated originally to be within the manufacturer's tolerance over the entire range. When an instrument is calibrated particularly for vitamin A, the wave-length scale will usually be a little bit less accurate in the visible range. It is definitely not satisfactory to use an instrument which has been calibrated with respect to the 656.3 mμ hydrogen tube lines.

2. Analysis of the mathematical formula for calculating the results of the U. S. P. XIV vitamin A assay leads to the fairly obvious data given in Table I.[1] It shows the effect of small errors at each wave length and also brings out the desirability of obtaining absorbancy readings in the higher positions of the useful range of the absorbancy scale.

3. Table I also gives emphasis to the point that the same error at all three wave lengths has no effect on the answer. For this reason *small* temperature differences have no effect on the answer, providing the temperature does not *change* during the time the readings at the three wave lengths are being made. We have found, however, that since the temperature of the cell compartment of the Beckman Spectrophotometer is usually higher than the room temperature, the solutions in the cells usually expand over a short period of time after the carrier is inserted in the compartment. This causes a small but definite decrease in absorption. Table II is typical of data we have gathered and has led to the general practice of allowing a waiting period before readings are attempted on a given solution in a cell so that there is no temperature change while a series of readings are being made.

4. Table III shows that there may be nonlinearity in the absorption of the cells themselves when one is checked against another with the same pure alcohol in each. Table IV shows the effect of failure to apply proper corrections. This effect is noted particularly with "Corex" cells (the use of which, for

[1] In this and subsequent tables, C denotes the amount of vitamin-A-bearing material per 100 cc. of isopropyl alcohol, and is one-tenth the value of C as defined in the U. S. P. XIV procedure. E denotes $E^{1\%}_{1\ cm.}$. $E_{corr.} \times 1{,}900$ equals U. S. P. potency.

TABLE III.—CELL DIFFERENCES FOR A TYPICAL "MATCHED" PAIR OF COREX ABSORPTION CELLS[a]

Wave Length, mμ	Absorbancy	Correction to Be Applied
300	−0.013	+0.013
305	−0.008	+0.008
310	−0.006	+0.006
315	−0.004	+0.004
320	−0.003	+0.003
325	−0.002	+0.002
330	−0.001	+0.001
334	−0.001	+0.001
340	−0.001	+0.001
345	−0.001	+0.001
350	−0.001	+0.001

[a] Absorbancy of one of the cells was determined against the other with the same pure isopropyl alcohol in both.

TABLE II.—EFFECT OF A CHANGE IN TEMPERATURE WHILE ABSORBANCY READINGS ARE BEING MADE[a]

	Reading at 1 Min.	2 Min.	3 Min.	4 Min.	5 Min.
A_{310}	0.616	0.614	0.614	0.614	0.614
A_{325}	0.725	0.723	0.722	0.722	0.722
A_{334}	0.645	0.644	0.644	0.644	0.644
$A_{corr.}$	0.6361	0.6318	0.6248	0.6248	0.6248
C	0.001014	0.001014	0.001014	0.001014	0.001014
$E_{corr.}$	627.3	623.1	616.2	616.2	616.2
% Error	1.80	1.12			

[a] Solutions were at room temperature when inserted in cell compartment. The effect varies directly with the difference between the cell compartment and the room temperature. The 325 mμ reading was taken first, then the 310 mμ, then the 334 mμ, and then repeated in that order. The times given are the times at the start of each series of readings.

TABLE IV.—EFFECT OF FAILURE TO APPLY THE CELL CORRECTION

	Actual Correct Absorbancies	Apparent Absorbancies Before Corrections	Apparent Absorbancies if Cells Reversed
A_{310}	0.614	0.608	0.620
A_{328}	0.722	0.720	0.724
A_{328}'	0.644	0.643	0.645
$A_{corr.}$	0.6248	0.6309	0.6186
C	0.001014	0.001014	0.001014
$E_{corr.}$	616.2	622.2	610.1
% Error		+0.97%	−0.99%

TABLE V.—ASSAY RESULTS FOR A SAMPLE OF SYNTHETIC VITAMIN A PALMITATE

Date of Assay	E_{310}	E_{328}	E_{328}	$E_{corr.}$	U. S. P. Potency
First day	610.6	719.5	639.7	635.3	1,207,000
First day	610.8	717.6	636.7	634.0	1,205,000
First day	611.3	719.4	637.8	640.7	1,217,000
First day	608.4	713.3	633.7	623.3	1,184,000
Fourth day	607.2	717.2	636.2	643.0	1,222,000
Fourth day	609.7	719.7	640.6	634.8	1,206,000
Fourth day	609.9	718.8	638.6	636.7	1,210,000
Fourth day	611.0	719.7	639.2	637.6	1,211,000
Sixth day	611.5	721.2	638.4	650.0	1,235,000
Sixth day	613.3	722.4	639.8	647.3	1,230,000

the vitamin A determination, is contrary to the U. S. P. procedure), but may also be noted with unmatched or dirty quartz cells. This same effect has also been noted, and may become quite large if two different batches of isopropyl alcohol are checked against each other. In this latter case the assay results would be affected if the analyst, under pressure of routine work, had alcohol from one lot in the blank cell and used alcohol from another lot to dilute a given sample.

5. Table V gives data on a sample of synthetic vitamin A-palmitate which is typical of data obtained in this laboratory.

CONCLUSIONS

The conclusions we have drawn from our studies of the precision of the assay (based on data from a wide variety of vitamin-A-bearing materials, including many pharmaceutical mixtures) are as follows:

1. That it is entirely possible, using the glassware and techniques described in this paper, to perform the saponification and extraction with a precision of ±1 per cent.

2. That with a stable spectrophotometer it is entirely possible for a careful and experienced operator to duplicate absorbancy readings on a given cell containing a vitamin A solution to 0.001 unit.

3. That it is entirely possible to obtain absorbancy readings at each wave length on replicate ether extractions of the same sample which are well within the precision of the saponification and extraction steps.

4. That if careful attention is paid to details, including the points mentioned in this paper, the combined errors of determining the absorbancies at the three wave lengths are not additive but, in general, tend to compensate for each other, and the corrected E values resulting can be obtained with a precision of at least ±3 per cent and often better.

REFERENCES

(1) "United States Pharmacopoeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950.
(2) Rawlings, H. W., and Wait, G. H., *Oil & Soap*, 23, 83(1946).

A Study of Folic Acid Stability in Solutions of the B Complex Vitamins*,†

By ALFRED R. BIAMONTE and GEORGE H. SCHNELLER

The stability of folic acid was studied at *p*H values between 3 and 7 in liquid media containing, individually and conjointly, the following members of the vitamin B complex: thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine, and pantothenyl alcohol. At the lower *p*H levels where water or sucrose syrup was used as vehicle, the folic acid was largely undissolved, whereas at the higher *p*H levels, the folic acid in the concentration used was completely dissolved. In a mixture of propylene glycol and water, the folic acid was completely dissolved throughout the entire *p*H range. Data are presented on the amount of folic acid decomposition after storage at room temperature and also at 45°. In general, riboflavin and thiamine caused considerable decomposition of folic acid in solution. Specimens at the lower *p*H levels where the folic acid was for the most part undissolved exhibited a stability which might be practical for pharmaceutical compounding purposes. Nicotinamide, pyridoxine, and pantothenyl alcohol did not materially affect the stability of folic acid in solution. Data are also presented on the solubility of folic acid in water as a function of *p*H and of temperature, and on the stability of folic acid in aqueous media as a function of *p*H. The decomposition of folic acid in the presence of riboflavin and thiamine involves cleavage at the methylene linkage, liberating an aromatic amine, presumably *p*-aminobenzoylglutamic acid. This is analogous to the reaction which takes place upon the acid reduction of folic acid as is used in the chemical determination of this substance.

DESPITE the wide interest in folic acid and the variety of products in which it occurs as an ingredient, published information regarding the pharmacy of this vitamin is extremely meager. The following data of pharmaceutical interest have appeared in biochemical and organic chemical reports, and merit collection in a publication of this scope.

The solubility of the *Lactobacillus casei* factor in water was reported by Stokstad (1) as approximately 1 mg./100 ml. at 2° (*p*H 3), and greater than 50 mg./100 ml. at 100°. He described the silver and lead salts as insoluble in water. Pfiffner (2) reported a solubility for vitamin B_c of 0.16 mg. (*p*H 3) at 25° and approximately 100 mg./100 ml. of boiling water. He further reported it to be insoluble in the common organic solvents, but relatively soluble in acetic acid, phenol, and pyridine. The zinc and silver salts were reported insoluble in water. Scheindlin (3) found that a pharmaceutical elixir, diluted to a concentration of 2.5 mg. of synthetic folic acid per 4 cc., precipitated after one week at *p*H 5.1, but remained clear at *p*H levels of 5.9 or higher. He further showed that stable solutions could be achieved in a concentration of 2.5 mg. or more per 4 cc. in the range of *p*H 8 to 9, even in the presence of as much as 50 per cent by volume of alcohol, glycerin, or propylene glycol. He reported the calcium and strontium salts to be insoluble and a clear, stable solution to be obtained with ferric ammonium citrate. Ferrous folate was also reported under certain conditions to be soluble (3) and stable (4). A 5 per cent solution of folic acid in sodium bicarbonate solution has been described (5).

The *p*H of the medium, and also the temperature, affect the chemical stability of folic acid. Daniel and Kline (6) found that autoclaving an aqueous solution of folic acid at 121° for thirty minutes effected the complete destruction of the compound at *p*H 1; at *p*H 3 there was 40 to 45 per cent decomposition; in the *p*H range 4 to 12, the amount of decomposition varied between 10 and 30 per cent. When liver extract preparations containing folic acid were treated in similar fashion, the results were much the same. On the other hand, Dick and coworkers (7) reported that negligible decomposition occurred on autoclaving folic acid solutions for fifteen minutes at 121° at *p*H 5 to 10; they also heated such solutions for one hour at 100° with similar results. Dick's study established that the thermal decomposition of folic acid in aqueous solution involves a unimolecular reaction, and that the decomposition velocity coefficient is directly proportional to the hydrogen-ion concentration.

Koft and Sevag (8) reported that aqueous solutions of folic acid on incubating at 30° during seven days underwent decomposition to the ex-

* Received January 25, 1951, from the Pharmaceutical Application Laboratory, American Cyanamid Co., Calco Chemical Division, Bound Brook, N. J.

† Presented at the joint session of the Subsection on Pharmacy, American Association for the Advancement of Science and the AMERICAN PHARMACEUTICAL ASSOCIATION, Scientific Section, Cleveland, Ohio, December 29, 1950.

tent of 3.9 per cent at *p*H 7.3 and 26 per cent at *p*H 9.2; at *p*H 4.5 the folic acid was incompletely dissolved (total concentration 0.5 mg./ml.) and the degree of decomposition was only 1.6 per cent. While folic acid stock solution prepared with the aid of alkali is known to be stable for three months at 4° (6), no extensive data have been published with regard to folic acid stability in aqueous solutions at room temperature or at slightly elevated temperatures.

Certain miscellaneous observations appearing in the literature are of pharmaceutical interest. An aerobic organism has been described, capable of converting folic acid to pteroic acid in a *p*H 7.0 phosphate buffer medium (9). Dilute folic acid solutions in water or 20 per cent ethanol were rapidly decomposed with the splitting off of *p*-aminobenzoylglutamic acid when exposed to daylight (10). This occurred at *p*H 7.0 (phosphate buffer) and also in 0.1 *N* HCl and 0.1 *N* NaOH. Artificial ultraviolet light also caused decomposition, but with less rapidity. Alkaline solutions were oxidized in the presence of oxygen with a similar type of cleavage (11). Likewise, reduction with zinc and acid liberates *p*-aminobenzoylglutamic acid (12); this is used as the basis of an assay procedure for folic acid (13). Sulfites (6, 14) cause decomposition, as do strong oxidizing and reducing agents in general (15). Folic acid reacts with nitrous acid to form N^{10}-nitrosopteroylglutamic acid (16).

Scheindlin (3) has reported on a study of the stability of folic acid under various conditions and its compatibility with a number of pharmaceutical chemicals, galenicals, and proprietary liquid products. In the latter group, an elixir of thiamine hydrochloride remained clear and did not show diminished folic acid activity after three months' storage at room temperature, but an elixir of vitamin B complex and a multivitamin syrup suffered a folic acid loss of more than 50 per cent. Another liquid vitamin B complex product showed separation, but did not decline in folic acid potency. After storage for five weeks at 42°, the results were somewhat different. The elixir of thiamine hydrochloride and the liquid vitamin B complex product retained full potency; the elixir of vitamin B complex declined in potency to the extent of 26 per cent, and the multivitamin syrup to the extent of 15 per cent.

It was the purpose of this investigation to study the stability of folic acid in solutions containing individually and conjointly the following vitamins of the B complex: thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, and pantothenyl alcohol. Another objective was to determine the influence of *p*H and temperature upon the solubility of folic acid in water, and also the effect of these factors and of the physical state upon the stability of folic acid in aqueous buffer solutions. The influence of several liquid vehicles upon the stability of this vitamin was also investigated.

EXPERIMENTAL AND RESULTS[1]

Effect of *p*H on the Solubility of Folic Acid.—The solubility of folic acid[2] at various *p*H levels was determined in McIlvaine's (17) buffers in the *p*H range 3 to 7. Saturated solutions of folic acid were prepared by agitating the buffer solutions (*p*H 3, 4, 5, 6, and 7) with an excess of folic acid in glass-stoppered vessels during one- and forty-eight-hour periods at 30° ± 2°. The solutions were filtered and the dissolved folic acid was determined by chemical analysis. The results shown in Fig. 1 indicate the solubility at the final *p*H attained in each solution.

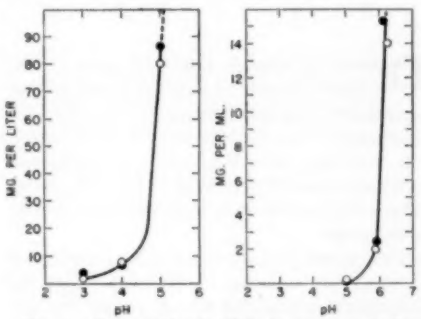


Fig. 1.—Folic acid solubility in aqueous buffers (sodium phosphate-citric acid). Temperature 30 ± 2° C. ○, one hour saturation period; ●, forty-eight-hour saturation period.

Influence of Temperature on the Solubility of Folic Acid.—Solutions of folic acid were prepared at temperatures ranging from 25 to 95°. Freshly boiled and cooled distilled water was stirred mechanically with an excess of folic acid during one hour, in a thermostatically controlled bath. The undissolved folic acid was removed by filtration at the corresponding temperature, and the filtrates were assayed chemically. The results shown in Fig. 2 illustrate graphically the change in solubility with temperature under the specified conditions. The solubility of folic acid was not determined at equilibrium conditions. A change in time employed for mixing folic acid in water may result in a different solubility, particularly at the higher temperatures. For example, folic acid and water at 80° were agitated for seventy-two hours and the resulting solubility was approximately 30% greater than that obtained after one-hour agitation.

[1] The analyses were performed by Mr. Harvey Hodes.
[2] U. S. P. folic acid was used throughout this study.

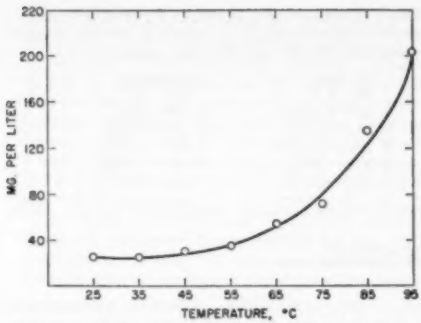


Fig. 2.—Folic acid solubility determined by stirring folic acid with water during one hour.

Stability of Folic Acid in an Aqueous Buffer System.—A series of samples was prepared (having a folic acid concentration of 1 mg./ml.) in sodium phosphate-citric acid buffer at *p*H levels between 3 and 9 and in boric acid-potassium chloride-sodium hydroxide buffer (13) at *p*H 10. In preparing each sample, 25 ml. of a stock solution containing 0.25 Gm. of folic acid was added to approximately 200 ml. of the buffer solution. Five milliliters of an alcoholic solution of methyl and propyl *p*-hydroxybenzoates (1.5% and 0.5%, respectively) was added as a preservative. The final volume was made up to 250 ml. with the buffer solution. When an adjustment in *p*H was necessary, sodium phosphate or citric acid was used. In the samples at the lower *p*H levels, part of the folic acid precipitated during the course of preparation. The latter samples were not filtered, but were carried through the stability test as mixtures. All samples were assayed chemically and then stored in the dark at room temperature, at 6° and also at 45°. These were reassayed after various periods of storage. The results are indicated in Table I.

The stock solution was prepared by dissolving folic acid at a concentration of 10 mg./ml. (real content) in a minimum quantity of 0.1 *N* sodium hydroxide. Methyl *p*-hydroxybenzoate (0.05%) and 0.01% of propyl *p*-hydroxybenzoate were added. The solution was made up to volume with distilled water after adjusting its *p*H to 7.

In order to study the stability of the folic acid in solution at the lower *p*H levels, a second group of specimens was prepared at *p*H 3 to 6, wherein the precipitated folic acid was filtered off. The specimen at *p*H 6 did not develop any immediate precipitate. The clear solutions were assayed before and after storage. The results are shown in Table II. It is apparent that aqueous solutions of folic acid at *p*H below 6 are subject to decomposition during storage.

TABLE II.—STABILITY OF SATURATED SOLUTIONS OF FOLIC ACID IN AQUEOUS BUFFERS[a]

	Folic Acid Recovered, Mg./Ml. and Per Cent Change		
*p*H	Initial Assay, 100%	21 Days, 45° ± 1°[b]	11 Months, 25° ± 5°
3.0	0.004	0.003 (−25%)	0.000 (−100%)
4.0	0.006	0.006 (0)	0.000 (−100%)
5.0	0.053	0.051 (−4%)	0.030 (−43%)
6.0[b]	1.02	1.11 (+9%)	1.030 (+1%)

[a] Solutions at *p*H 3 to 5 are saturated solutions.
[b] Made to contain 1 mg. folic acid per ml.

Stability of Folic Acid in Syrup.—A series of samples was prepared identical with those in the previous group except for the inclusion of sucrose. Those specimens which developed a precipitate of folic acid were not filtered but were studied as mixtures. The sugar content was 75 Gm./100 ml. The stability of these samples after storage was essentially the same as that shown in Table I for samples made without sucrose, except in the samples containing sucrose at *p*H 5.4 the folic acid did not precipitate, whereas in those made without sucrose it had precipitated.

Stability of Folic Acid with B Complex Vitamins in an Aqueous Buffer System.—Samples were prepared at *p*H levels between 3 and 7 in sodium phosphate-citric acid buffer solutions. Each 5 cc. was made to contain:

Folic acid (real)	1.66 mg.
Riboflavin	1.0 mg.
Thiamine hydrochloride	1.0 mg.
Nicotinamide	10.0 mg.
Methyl *p*-hydroxybenzoate	2.5 mg.
Propyl *p*-hydroxybenzoate	0.5 mg.
Sodium phosphate-citric acid buffer *q. s.*	

TABLE I.—STABILITY OF FOLIC ACID IN AN AQUEOUS BUFFER SYSTEM[a]

		Folic Acid Recovered, Mg./Ml.				
*p*H	Initial Description	Initial Assay	21 Days, 45° ± 1°[b]	4 Months, 25° ± 5°	18 Months, 25° ± 5°	18 Months, 6° ± 2°
2.5	Mixture[b]	0.89	0.98	1.03	0.98	1.06
3.3	Mixture[b]	1.00	0.92	1.00	0.98	1.08
4.3	Mixture[b]	1.04	0.93	1.00	0.91	1.05
5.3	Mixture[b]	1.04	0.93	1.00	0.91	1.05
5.5	Mixture[b]	1.06	0.95	1.03	0.89	1.01
5.6	Mixture[b]	0.93	0.95	0.99	0.84	1.02
6.9	Solution[c]	0.87	0.93	0.98	0.68[d]	1.00
7.9	Solution[c]	0.91	0.93	0.98	0.85	0.99
8.4	Solution[c]	0.91	0.98	1.01	0.92	1.02
9.8[e]	Solution[c]	1.00	0.99	0.98	0.73	1.04

[a] Samples were made to contain 1 mg. folic acid per ml.
[b] Contains a sediment of folic acid because its solubility has been exceeded.
[c] Developed a precipitate during storage.
[d] This result considered anomalous.
[e] Clark and Lubs' buffer mixture; all others contained McIlvaine's buffers.

TABLE III.—STABILITY OF FOLIC ACID WITH B COMPLEX VITAMINS IN AN AQUEOUS BUFFER SYSTEM[a]

		Folic Acid Recovered, Mg./Ml. and Per Cent Change		
pH	Initial Description	Initial Assay, 100%	25 Days, 45° ± 1°	16 Months, 25° ± 5°
3.2	Mixture[b]	0.32	0.28 (−16%)	0.33 (+3%)
4.0	Mixture[b]	0.33	0.26 (−21%)	0.34 (+3%)
5.0	Mixture[b]	0.33	0.10 (−70%)	0.07 (−80%)
6.0	Solution[c]	0.23[d]	0.05 (−78%)	0.05 (−78%)
7.0	Solution[c]	0.29[d]	0.04 (−86%)	0.03 (−90%)

[a] Composition—Each ml. contains: folic acid, 0.33 mg.; riboflavin, 0.2 mg.; thiamine hydrochloride, 0.2 mg.; nicotinamide, 2.0 mg.; McIlvaine's buffers *q. s.*
[b] Contains undissolved folic acid.
[c] Solutions were clear when prepared; developed a precipitate during storage.
[d] Samples were filtered when prepared. This may account for low initial assay values.

Riboflavin was added to a portion of the buffer vehicle in which the nicotinamide had been previously dissolved. The mixture was heated to 80° to effect complete solution of the riboflavin. The solution was cooled and the thiamine hydrochloride was added, followed by the preservatives and the folic acid. The folic acid did not dissolve completely at the lower *p*H levels. The samples were assayed for folic acid before and after storage. The results obtained are listed in Table III.

Stability of Folic Acid with B Complex Vitamins in Syrup.—The formula of this series is identical with the previous group except that the product contained 80 Gm. of sucrose per 100 ml. Table IV presents the results obtained before and after storage.

Stability of Folic Acid with B Complex Vitamins in Propylene Glycol-Syrup Mixture.—For this series of experiments two groups of samples were prepared at *p*H levels between 3 and 7. The vitamin content was the same as in the previous group. The major difference was in the vehicles used, namely, group A contains 48% sucrose and 25% propylene glycol in aqueous sodium phosphate-citric acid buffer, while B contains 10% sucrose and 75% propylene glycol in diluted buffer. A reduction of the buffer salts was necessary in the latter groups because, when used in full quantities, the buffer salts tended to crystallize. The specimens containing 25% propylene glycol formed the usual sediment of precipitated folic acid at the lower *p*H levels, whereas those containing 75% propylene glycol were clear solutions throughout the *p*H range studied. Both groups of specimens were assayed for folic acid before and after aging. The results obtained are presented in Table V.

Stability of Folic Acid with Thiamine Hydrochloride in Aqueous Buffers.—Samples were prepared at *p*H levels between 3 and 7 in sodium phosphate-citric acid buffer. Thiamine hydrochloride (1 mg./ml.) was dissolved in the buffer solutions. Crystalline folic acid was added at a concentration of 1 mg./ml. Preservative was added and the samples were diluted to volume with the corresponding buffer. The samples were agitated for at least one hour (at room temperature) to aid the solution of folic acid. The well-mixed samples were assayed before and after storage. The results shown in Table VI show appreciable destruction of folic acid at *p*H 6 and 7. Tests were made at these *p*H levels because of the greater solubility of folic acid, despite the fact that *p*H 6 and 7 are considered unfavorable for thiamine stability. Folic acid-thiamine mixtures at *p*H levels between 3 and 5 (wherein folic acid is for the most part undissolved) showed good folic acid stability during three weeks' storage at 45°. High folic acid assay values were obtained at *p*H 3 to 5 after storage for eleven months at room temperature. A satisfactory explanation for these high assays has not been elicited, although mold growth was observed in these samples and may have been responsible for the erratic results. It is also possible that decomposition or interaction products resulting from thiamine and folic acid caused color interference during analysis.

Stability of Folic Acid with Riboflavin, Nicotinamide, Pyridoxine Hydrochloride, and Pantothenyl Alcohol in Aqueous Buffers.—Similar series of samples were prepared (containing 1 mg. folic acid per ml.) with each of the following vitamins: riboflavin, nicotinamide, pyridoxine hydrochloride, and pantothenyl alcohol.

The folic acid-riboflavin samples were made to contain 1 mg. of riboflavin per 5 ml. Stability results are shown in Table VII. Almost total decomposition of folic acid occurred during nine months' storage at room temperature in solutions at *p*H 5.9 and 6.9. Mixtures containing undis-

TABLE IV.—STABILITY OF FOLIC ACID WITH B COMPLEX VITAMINS IN SYRUP[a, b]

		Folic Acid Recovered, Mg./Ml. and Per Cent Loss			
pH	Initial Description	Initial Assay, 100%	3 Weeks, 45° ± 1°[b]	6 Weeks, 45° ± 1°[b]	15 Months, 25° ± 5°
3.3	Mixture[c]	0.32	0.31 (−3%)	0.29 (−9%)	0.30 (−6%)
4.0	Mixture[c]	0.32	0.30 (−3%)	0.27 (−16%)	0.31 (−3%)
4.9	Mixture[c]	0.30	0.17 (−43%)	0.13 (−56%)	0.19 (−37%)
5.5	Solution[d]	0.29	0.17 (−44%)	0.12 (−59%)	0.06 (−80%)
6.4	Solution[d]	0.33	0.19 (−33%)	0.12 (−63%)	0.05 (−85%)

[a] Composition—Each ml. contains: folic acid, 0.33 mg.; riboflavin, 0.2 mg.; thiamine HCl, 0.2 mg.; nicotinamide, 2.0 mg.; syrup *q. s.*
[b] 80 Gm. sucrose/100 cc
[c] Contains undissolved folic acid.
[d] Free from sediment during storage.

2502

solved folic acid were stable at pH 3 and 4. At pH 5 a 25% loss in folic acid had occurred.

The folic acid-nicotinamide samples contained 25 mg. of nicotinamide per ml. The results obtained indicated good folic acid stability after ten months' storage at room temperature. Mixtures (containing undissolved folic acid) at pH between 3 and 5 underwent no change, while solutions at pH 6 and 7 showed a loss in folic acid of only 8% and 7%, respectively.

The folic acid-pyridoxine specimens contained 5 mg. of pyridoxine hydrochloride per ml. After ten months' storage at room temperature, these samples showed negligible decomposition between pH 3 and 5 (mixtures) while at pH 6 and 7 (solutions) a loss of 13% and 8%, respectively, was observed. During the twenty-one-day 45° test period, 18% and 3% loss in folic acid was noted in solutions having pH 6 and 7, respectively, while the decomposition of folic acid in mixtures at pH 3 to 5 was insignificant.

For the samples containing pantothenyl alcohol, 5 mg. of that substance was used per ml. In the pH range 3 to 5, the samples (mixtures) demonstrated good folic acid stability after storage for twenty-one days at 45° and after eleven months at 25°. Solutions at pH 6 and 7, after accelerated storage, had lost 5% and 3% folic acid, respectively, whereas

TABLE V.—STABILITY OF FOLIC ACID WITH B COMPLEX VITAMINS IN PROPYLENE GLYCOL-SYRUP MIXTURE[a]

A. Propylene Glycol 25%, Syrup 75%[b]

pH	Initial Description	Initial Assay, 100%	Folic Acid Recovered, Mg./Ml. and Per Cent Loss: 24 Days, 45° ⇌ 1°	45 Days, 45° ⇌ 1°	14 Months, 25° ⇌ 5°
3.4	Mixture[c]	0.31	0.29 (−6%)	0.27 (−13%)	0.27 (−13%)
4.3	Mixture[c]	0.29	0.25 (−14%)	0.21 (−28%)	0.25 (−14%)
5.2	Mixture[c]	0.28	0.17 (−39%)	0.11 (−60%)	0.10 (−64%)
6.3	Solution	0.30	0.19 (−37%)	0.13 (−56%)	0.06 (−80%)

B. Propylene Glycol 75%, Syrup 25%[d]

pH	Initial Description	Initial[e] Assay, 100%	Folic Acid Recovered, Mg./Ml. and Per Cent Loss: 12 Days, 45° ⇌ 1°	45 Days, 45° ⇌ 1°	15 Months, 25° ⇌ 5°
3.0	Solution	0.20	0.10 (−50%)	0.03 (−85%)	0.04 (−80%)
4.1	Solution	0.18	0.10 (−44%)	0.03 (−83%)	0.05 (−72%)
4.9	Solution	0.18	0.12 (−33%)	0.03 (−83%)	0.03 (−83%)
6.2	Solution	0.20	0.17 (−15%)	0.05 (−75%)	0.10 (−50%)

[a] Composition—Each ml. contains: folic acid, 0.33 mg.; riboflavin, 0.2 mg.; thiamine hydrochloride, 0.2 mg.; nicotinamide, 2.0 mg.; propylene glycol-syrup *q. s.*
[b] Sugar content, 48 Gm./100 ml.
[c] Contains undissolved folic acid.
[d] Sugar content, 10 Gm./100 ml.
[e] Initial assays were performed after storing the samples for five days at 6°. Average initial loss of folic acid was 43%.

TABLE VI.—STABILITY OF FOLIC ACID WITH THIAMINE HYDROCHLORIDE IN AQUEOUS BUFFERS[a]

pH	Initial Description	Initial Assay, 100%	Folic Acid Recovered, Mg./Ml. and Per Cent Change: 21 Days, 45° ⇌ 1°	11 Months,[b] 25° ⇌ 5°
3.1	Mixture[c]	1.02	1.05 (+3%)	1.23 (+22%)[e]
4.0	Mixture[c]	1.03	1.04 (+1%)	1.43 (+38%)[e]
5.0	Mixture[c]	1.06	1.05 (−1%)	1.27 (+20%)[e]
5.9	Solution[d]	1.04	0.93 (−11%)	0.63 (−40%)
6.9	Solution	1.04	0.90 (−14%)	0.56 (−46%)

[a] Composition—Each ml. contains: folic acid, 1 mg., thiamine HCl, 1 mg., McIlvaine's buffer *q. s.*
[b] These specimens contained mold growths.
[c] Contains undissolved folic acid.
[d] Developed precipitate during storage.
[e] High assay values may be the result of interference during analysis, due to extraneous colors caused possibly by mold growth and from decomposition or interaction products of thiamine and folic acid.

TABLE VII.—STABILITY OF FOLIC ACID WITH RIBOFLAVIN IN AQUEOUS BUFFERS[a]

pH	Initial Description	Initial Assay, 100%	Folic Acid Recovered, Mg./Ml. and Per Cent Loss: 21 Days, 45°	9 Months, 25° ⇌ 5°
3.0	Mixture[b]	1.00	0.94 (−6%)	0.98 (−2%)
4.0	Mixture[b]	1.01	0.95 (−6%)	0.98 (−2%)
5.0	Mixture[b]	1.06	0.45 (−58%)	0.79 (−25%)
5.9	Solution[c]	1.06	0.23 (−78%)	0.11 (−90%)
6.9	Solution[c]	1.06	0.16 (−85%)	0.06 (−94%)

[a] Composition—Each ml. contains: folic acid, 1 mg., riboflavin, 0.2 mg., McIlvaine's buffer *q. s.*
[b] Contains undissolved folic acid.
[c] Clear solution when prepared. Developed a precipitate during storage.

after eleven months' storage at room temperature, 17% decomposition was observed at both *p*H levels

ANALYTICAL PROCEDURE

The analytical procedure used in these experiments is based upon the method developed by Hutchings, *et al.* (12, 13), with certain modifications proposed by Seaman and co-workers (18), and others by Woolford and Avery (19), and with some additional changes. Ware and Cronheim (20) have also described modifications of the Hutchings procedure.

The method involves the reduction of the folic acid with zinc amalgam and an acid, to yield a pteridine and *p*-aminobenzoylglutamic acid (PABG). The liberated PABG is determined by the method of Bratton and Marshall (21), involving diazotization and treating with a coupler to develop a color.

Considering the reproducibility of the assay procedure and the problem of obtaining a representative sample in those preparations containing undissolved folic acid or other precipitated matter, variations in assay values within the limits of ±5% were arbitrarily regarded as insignificant. All samples were assayed directly, without any preliminary separation or extraction of the folic acid from the other constituents.

In some instances, such as those involving samples containing riboflavin, it was noted that a significant amount of folic acid decomposition occurred during the preparation of the solution for assay. This decomposition was minimized by maintaining the solution in an ice bath and by completing the analysis as rapidly as possible.

Pipette a representative sample equivalent to approximately 5 mg. of folic acid into a 250-ml. volumetric flask. Add 20 ml. of 0.1 *N* NaOH to dissolve any precipitated folic acid, followed by 25 ml. of 5 *N* HCl, and dilute to volume with distilled water. (Represents solution A.) It is at this point in the procedure that folic acid may decompose in the presence of riboflavin or thiamine hydrochloride. Therefore, the analysis should be continued with a minimum loss of time. Placing this solution in an ice bath will retard the decomposition.

To prepare the unreduced solutions, transfer 25-ml. portions (from solution A) into each of two 100-ml. volumetric flasks (flasks 1 and 2). Add 30 ml. HCl (1:5) and mix well. Add to each flask 10 ml. of sodium nitrite, 1% solution. Agitate the solutions during five minutes, then add to each flask 10 ml. of a 5% solution of ammonium sulfamate. Shake the flasks vigorously to expel the liberated gas, taking care to avoid losses by spattering. Continue the agitation during three minutes. To flask 2, add 10 ml. of a 0.1% solution of N-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1% solution. Dilute flasks 1 and 2 to volume. Agitate them intermittently during ten minutes. The color produced is measured at 550 mμ in a Beckman Spectrophotometer.

To prepare the reduced test solutions, transfer approximately 65 ml. of solution A to a strong screw-cap bottle of approximately 200 ml. capacity. Add 10 ml. of zinc amalgam made from 10 Gm. of mossy zinc for each 20 ml. of mercury. Stopper the bottle tightly and agitate for thirty minutes. (Designated as solution B.) Transfer 25 ml. of the reduced solution (solution B) to each of two 100-ml. volumetric flasks (flasks 3 and 4). Continue the assay as stated above, starting with, "add 30 ml. HCl (1:5) etc.," the coupling reagent being added only to flask 4.

A standard curve is prepared by diazotizing and coupling a series of *p*-aminobenzoylglutamic acid (PABG) solutions as described above. The per cent transmission obtained for each solution in flasks 1 to 4 inclusive, is read on the standard curve, and the resulting concentration of PABG is obtained for each solution. Folic acid is determined by the following formula:

$$\left[\begin{matrix}\text{Solution B}\\ \text{Reduced Sample}-\text{Reduced Blank}\\ (4-3)\end{matrix}\right] - \left[\begin{matrix}\text{Solution A}\\ \text{Unreduced Sample}-\text{Unreduced Blank}\\ (2-1)\end{matrix}\right] = \text{PABG, equivalent to folic acid in the diluted sample}$$

(Numbers refer to the numbered flasks wherein the PABG found in each solution is substituted.) The weight of PABG obtained is multiplied by the factor 1.66 (mol. wt. folic acid/mol. wt. PABG) to convert it to folic acid. This represents the folic acid in the diluted sample. The use of appropriate dilution factors gives the folic acid content in the original material.

The reduced and unreduced blanks are included in order to correct for the color caused by extraneous substances, in both the reduced and unreduced samples.

DISCUSSION

The solubility of folic acid in aqueous systems is greatly dependent upon *p*H. At *p*H 5.6 or higher, the vitamin is sufficiently soluble to permit the preparation of solutions sufficiently concentrated for many pharmaceutical purposes. The studies herein reported dealt with preparations containing 1 mg./ml. and this amount of folic acid was completely dissolved at approximately *p*H 5.6 or higher. At *p*H 5.0, 1 mg./ml. of folic acid was not completely dissolved, and in the range *p*H 3 to 4 the folic acid remained substantially undissolved. The concentration of 1 mg./ml. was selected as representing the center, more or less, of pharmaceutical interest. Such preparations would contain 5 mg. of folic acid in an average teaspoonful. It is obvious that elixirs or syrups containing folic acid alone in such a concentration are feasible from the point of view of solubility. In other types of products, such as in parenteral solutions, a higher concentration might be desirable, and here again it can be readily achieved, merely by raising the *p*H to a suitable level. However, there are many possible combinations wherein it is necessary, for the sake of the stability of other ingredients, to have a low *p*H level, such as 3 to 4 as required in multivitamin products. In this range the solubility of folic acid is very low, and folic acid, if added in the concentration mentioned, would remain largely undissolved. This, of course, does not eliminate the possibility of formulating such products containing

folic acid. In the first place, the undissolved folic acid might be suspended with due pharmaceutical elegance, and this problem is aided in the case of many liquid multivitamin and hematinic preparations by the high viscosity, low surface tension, and often, dark color, of the basic product. Furthermore, as will be discussed below, the fact that the folic acid is present undissolved may actually protect it from deleterious influences exerted by other components of the preparation.

The stability of folic acid in aqueous media is in general very good. The data (Table I) show that dissolved folic acid at *p*H 6.0 or higher is stable for one year at room temperature. At the lower *p*H levels (3 to 4) the very small amount of folic acid which goes into solution is unstable (Table II) but mixtures containing undissolved folic acid at concentrations exceeding its solubility exhibit good stability (Table I) and are pharmaceutically practicable and useful.

Riboflavin and, to a lesser degree, thiamine cause the decomposition of dissolved folic acid. This decomposition is fairly rapid so that a significant amount can occur even during the preparation of a sample for analysis. In the gross preparations, loss of folic acid was most evident in the *p*H range 6 and 7, where the folic acid was completely dissolved. At *p*H 5, where the folic acid was partly undissolved, the decomposition was less, and at *p*H 3 and 4, where the folic acid was essentially undissolved, good stability was exhibited. The latter results indicate that folic acid may be compounded in liquid preparations at *p*H levels designed for optimum stability of the vitamins of the B complex, the problem being chiefly one of achieving a satisfactory homogeneity of suspension of the folic acid.

Pyridoxine and pantothenyl alcohol caused some decomposition of dissolved folic acid in certain samples. However, this effect was not sufficient to present a serious compatibility problem in itself. Samples at low *p*H levels containing undissolved folic acid were not affected by pyridoxine or pantothenyl alcohol. Nicotinamide was entirely innocuous at all *p*H levels.

Among the vehicles tested in the multivitamin group, the samples containing propylene glycol exhibited the greatest amount of folic acid decomposition. The vehicle containing sucrose did not exhibit any significant improvement in stability when compared with that obtained with aqueous buffers alone.

The high degree of stability of undissolved folic acid in aqueous media containing dissolved B complex vitamins might be used as a basis for the prediction that the manufacture by ordinary methods, and the storage of B complex vitamin tablets and capsules, would not be fraught with special difficulties related to folic acid stability.

It is well known that reduction of folic acid in an acid medium results in the formation of a pteridine and *p*-aminobenzoylglutamic acid (12, 13). The decomposition of folic acid at low *p*H levels and also in the presence of other B complex vitamins involves a similar reaction. This fact became apparent in the course of the analyses of the experimental samples. The analytical procedure as described above involves the estimation of the aromatic amine *p*-aminobenzoylglutamic acid (PABG) before and after reduction of the folic acid with zinc amalgam. The PABG (free) content of the unreduced sample corresponds to the amount of folic acid which has undergone decomposition. The PABG liberated by reduction with zinc amalgam corresponds to the amount of undecomposed folic acid remaining in the sample before analysis. In general, there was good agreement between the two figures. That is to say, the sum of the free PABG present in the sample before analysis and the PABG liberated by the reduction with zinc amalgam, was usually approximately equal to the total PABG expected on the basis of the initial folic acid concentration. This point is illustrated in Fig. 3. The latter deals with a series of samples containing folic acid and riboflavin, and shows that as the folic acid content in the series declined, the free PABG content, calculated as folic acid, rose proportionately, so that the sum of the two at any point was approximately equal to the initial folic acid concentration.

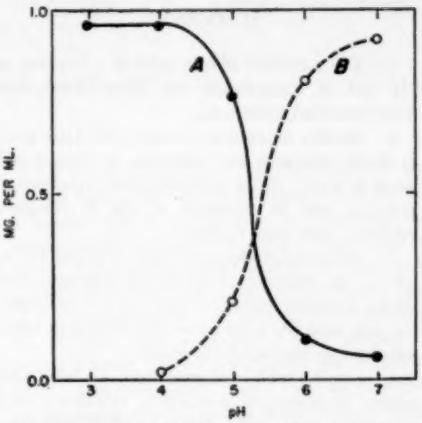


Fig. 3.—Folic acid-riboflavin: relationship between folic acid decomposition and liberation of *para*-aminobenzoyl glutamic acid (PABG). Assay values after nine months' storage at room temperature.

Initial composition: Each ml. contains folic acid, 1.0 mg.; riboflavin, 0.2 mg.; McIlvaine's buffer, *q. s.*

A, folic acid remaining; *B*, PABG resulting from folic acid decomposition (calculated as folic acid).

Folic acid stability data presented in this paper are based upon experiments performed with certain specified quantities of folic acid and other B vitamins. It is reasonable to expect that where decomposition of folic acid occurred with thiamine or riboflavin alone or in mixtures, the rate and amount of such decomposition would be dependent, among other factors, upon the concentration of these vitamins, i. e., for formulations having different concentration ratios of folic acid and thiamine or riboflavin, varying rates of folic acid decomposition would be expected. Likewise, while little or no decomposition was encountered with nicotinamide, pyridoxine, or pantothenyl alcohol at the concentrations used, the use of greater proportions of these vitamins in combination with folic acid might induce a significant degree of decomposition.

SUMMARY

1. The solubility of folic acid as a function of *p*H and of temperature has been determined under specified conditions.

2. Results of stability studies with folic acid, in liquid mixtures and solutions at several *p*H levels in water, syrup and propylene glycol-water vehicles, and in solutions of the B complex vitamins, have been reported.

3. Folic acid in aqueous solution at *p*H 6.0 to 9.8 in the presence of citrate-phosphate buffer shows excellent stability. At *p*H 5.0 or lower, the folic acid has a very low solubility, and these solutions decline rapidly in potency.

4. Considerable decomposition of folic acid occurs in solutions containing riboflavin and somewhat less with thiamine hydrochloride. Pantothenyl alcohol, pyridoxine hydrochloride, and nicotinamide (at the concentrations studied) had insufficient influence on the stability of the folic acid to be classified as incompatible substances.

5. Crystalline folic acid in an aqueous medium at *p*H 3 to 4 is stable in the presence of the B complex vitamins including thiamine and riboflavin. This suggests an approach to the successful compounding of folic acid with these vitamins in liquid mixtures or suspensions.

6. The decomposition of solutions of folic acid in the presence of other B complex vitamins is accompanied by the liberation of an equivalent amount of an aromatic amine, presumably *p*-aminobenzoylglutamic acid. This is analogous to the reaction used in the analysis of folic acid, involving reduction with zinc in the presence of an acid.

REFERENCES

(1) Stokstad, E. L. R., *et al.*, *J. Am. Chem. Soc.*, **70**, 3(1948).
(2) Pfiffner, J. J., *et al.*, *ibid.*, **69**, 1476(1947).
(3) Scheindlin, S., *Am. J. Pharm.*, **120**, 103(1948).
(4) Anicchio, G., *Boll. soc. ital. biol. sper.*, **23**, 361(1947).
(5) Harned, B. K., *et al.*, *Ann. N. Y. Acad. Sci.*, **48**, 289 (1946).
(6) Daniel, E. P., and Kline, O. L., *J. Biol. Chem.*, **170**, 739(1947).
(7) Dick, M. I. B., *et al.*, *Australian J. Exptl. Biol. Med. Sci.*, **26**, 239(1948).
(8) Koft, B., and Sevag, M. G., *J. Am. Chem. Soc.*, **71**, 3245(1949).
(9) Lemon, J., *et al.*, *Arch. Biochem.*, **19**, 311(1948).
(10) Stokstad, E. L. R., *et al.*, *J. Biol. Chem.*, **167**, 877 (1947).
(11) Stokstad, E. L. R., *et al.*, *J. Am. Chem. Soc.*, **70**, 5 (1948).
(12) Hutchings, B. L., *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).
(13) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950.
(14) Waller, C. W., *et al.*, *J. Am. Chem. Soc.*, **70**, 22(1948).
(15) Mitchell, H. K., and Williams, R. J., *ibid.*, **66**, 271 (1944).
(16) Cosulich, D. B., and Smith, J. M., Jr., *ibid.*, **71**, 3574 (1949).
(17) "Handbook of Chemistry and Physics," 13th ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1947.
(18) Seaman, W., and co-workers, unpublished data, Calco Chemical Division, American Cyanamid Co.
(19) Woolford, M. H., and Avery, M. E., unpublished data, American Cyanamid Co., Lederle Laboratories Division.
(20) Ware, P. A., and Cronheim, G., THIS JOURNAL, **39**, 98(1950).
(21) Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537(1939).

American Association for the Advancement of Science—Pharmacy Subsection Meeting

The next meeting of the American Association for the Advancement of Science will be held in Philadelphia on December 26–31, 1951. The program of the Pharmacy Subsection of the Medical Sciences is being made up. Six sessions of this Subsection will be held. Members are invited to attend the meetings of the Section on Medical Sciences, Subsection on Medicine, and the Section on Chemistry.

Arrangements will be made for advance registration. Advance registrants will be sent a copy of the General Program before the meeting. See *Science* or *Scientific Monthly* in late August or September.

Authors who wish to present papers before the Subsection are requested to send in titles of papers *as soon as possible*. All titles for inclusion in the printed program must be in the hands of Glenn L. Jenkins, Chairman, Subsection Np, Purdue University, School of Pharmacy, Lafayette, Ind., by *September 15*.

Antifungal Activity of a Series of Thiocyanates*

By L. LANDIS, D. KLEY, and N. ERCOLI†

The activity of more than 100 aryl oxyalkyl derivatives of thiocyanates and isothiocyanates against fungi pathogenic to humans has been determined. Of the entire series of compounds studied, γ(p-bromo)phenoxypropyl thiocyanate was found to be most active as an antifungal agent.

WALTON, Herbold, and Lindegren (1), in 1936, reported that the vapors from crushed garlic showed bactericidal activity. In 1937, Lovell (2) studied the same properties from vapors of crushed onions. Foter and Golick (3), in 1938, showed that the vapors from crushed horseradish roots similarly exerted a strong effect against several bacterial strains. Having rereviewed the chemical analyses of crushed horseradish by Goldmeister and Hoffman (1916) and Heiduschka and Zwergal (1931), who found that the main volatile components of horse-radish were mustard oil, allyl isothiocyanate, phenyl ethyl isothiocyanate, and phenyl propyl isothiocyanate, Foter (4) reported in 1940 on the bactericidal effect of the main constituent—allyl isothiocyanate—and two other oils of similar structure, methyl and ethyl isothiocyanates.

In surveying the antifungal activity of various organic compounds, it was noted that allyl isothiocyanate was quite effective against a number of fungi associated with dermatophytosis. This observation is in agreement with the findings of Walker, Morell, and Foster (5), who, in 1937, reported that the type of radical attached to the SCN or NCS group of the molecule of various mustard oils affected their toxicity to different microorganisms in the following descending order: allyl, phenyl, methyl, and ethyl. In 1943, Hooker, Walker, and Smith (6), also found that allyl isothiocyanate was toxic to several species of fungi. In a more recent publication (1946), Davies and Sexton (7) reported that thiocyanic and isothiocyanic acid derivatives and certain substituted aryl isothiocyanates have fungistatic activity. It is well known, however, that all of these compounds possess highly irritating properties which make them unsuitable for therapeutic use.

We have investigated the activity of aryl oxyalkyl derivatives of thiocyanates and isothiocyanates with the objective of preparing one which would be effective against a representative group of pathogenic fungi, and at the same time devoid of any unacceptable characteristics such as: unpleasant odor, lacrimation, and irritation. In all, over 100 preparations were studied of which a series of 20 thiocyanates are described here. The compounds tested were all prepared in the chemical laboratories of the Warner Institute (Genzer, Huttrer, and Van Wessem, to be published in *J. Am. Chem. Soc.*, July, 1951).

METHODS

In this study, our primary aim was not to formulate any new criteria for methods of testing. Those available were found satisfactory for the present investigation. For comparative purposes all test materials were subjected to the Burlingame and Reddish (8) and to the Golden and Oster (9) methods. The similarity of results with the two techniques has been previously reported by other workers. Hence, the methods we adopted for study were basically those of Burlingame and Reddish (8), with the following modifications:

(*a*) For fungistatic (solution and ointment) testing, as in all other cases, a ten- to twelve-day-old culture was used throughout.

(*b*) For fungicidal (solution) testing, culture disks were exposed for one, five-, ten-, and thirty-minute intervals; washings were made in 10 cc. of 25% aqueous ethyl alcohol.

In the course of our work, it was found that the results were not influenced to any great extent by the longer exposure periods; one- and five-minute contacts of compound and organism were sufficient to give maximum results possible with the drug concentrations used.

The procedures for sporostatic activity were as follows: 0.1 cc. of test solution was added to 20 cc. of Sabouraud's maltose agar (45°). Upon hardening, 0.1 cc. of a suspension containing about one million spores, counted with a Levy hemocytometer, was streaked over the agar surface. The spore suspensions were prepared by adding 10 cc. of saline to sporulating agar slants and scraping the surface growth with swabs. Intermittently, the dilution technique was also used. The growth inhibition activity of the compound was determined in serial dilution, using a sporulating medium[1] which gives

* Received May 5, 1950, from the Warner Institute for Therapeutic Research, New York, N. Y.
Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.
† Present address: Istituto Sieroterapico Milanese, Milano, Italy.

[1] Medium: 7.5 Gm. glycerin; 5.0 Gm. glucose; 5.0 Gm. peptone; 4.0 Gm. sodium chloride; 0.05 Gm. magnesium sulfate ($7.H_2O$); 0.06 Gm. potassium biphosphate; 0.003 Gm. ferric chloride; 0.001 Gm. copper sulfate; and 1.4 L. distilled water.

TABLE I.—ACTIVITY OF γ(*p*-BROMO)PHENOXYPROPYL THIOCYANATE

Test	Test Culture	1:100	1:200	1:300	1:400	1:500	1:750	1:1,000	1:1,600	1:2,000	1:3,000	1:4,000	1:5,000	1:10,000	1:20,000	1:40,000	1:60,000	1:80,000	1:90,000	1:100,000	1:125,000
A⁻¹	*T. mentagrophytes*	O	O	O	O	P	P	P													
	T. purpureum	O	O	O	O	P	P	P													
	E. floccosum	O	O	O	O	O	O	O													
B	*T. mentagrophytes*	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	P	P	P	I	I
	C. neoformans	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	I	I	I	I	I
	E. floccosum	O	O	O	O	O	O	O	O	O	O	O	P	I	I	I	I	I	I	I	I
C	*T. mentagrophytes*	O	O	O	O	I	I	I	I	I	I	O	O	O	O	O	O	O	O	O	I
	T. purpureum	O	O	O	O	I	I	I	I	I	I	O	O	O	O	O	O	O	O	O	I
	E. floccosum	O	O	O	O	I	I	I	I	I	I	O	O	O	O	O	O	O	O	O	I
	C. neoformans	O	O	O	O	I	I	I	I	I	I	O	O	O	O	O	O	O	O	O	I
D	*A. fumigatus*	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	I
	T. mentagrophytes	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	I
	T. purpureum	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	I

NOTE: O = active; P = partially active; I = inactive; A⁻¹ = fungicidal; B = fungistatic (solution); C = fungistatic (ointment); D = sporostatic.

abundant growth within two days. A twofold serial dilution of the test preparation was made in 0.8-cc. amounts of medium with 0.2 cc. of a spore suspension (30,000/tube) added to all tubes, including controls.

Observations on all experiments, except the serial dilution, were for periods of about three weeks. The organisms used interchangeably were: *Trichophyton mentagrophytes* (Emmons No. 640), *Trichophyton purpureum* (*Epidermophyton rubrum*), *Epidermophyton floccosum* (inguinale), *Cryptococcus neoformans*, *Microsporum felineum*, and *Aspergillus fumigatus*. All of the compounds were water insoluble; 95% ethyl alcohol was used throughout as solvent. Control tests on the effect of the solvent, as well as on commercial preparations (Table III), were performed simultaneously.

RESULTS

The activity was determined by establishing the minimal effective concentration for each compound. To illustrate how these figures were obtained, the experiments on γ(*p*-bromo)phenoxypropyl thiocyanate are presented fully in Table I. Each of the preparations was treated in a similar manner and, in addition, was treated repeatedly with other techniques in order to obtain a better indication of activity. The results tabulated, however, are all based on the modified procedures adapted for our own purposes. It appears that the sensitivity of the fungal strains studied does not vary widely in many of the accepted techniques. In general, the relative value of different preparations closely paralleled the various strains and procedures. The method evolved by Schamberg and Kolmer (10) appears in our hands somewhat less sensitive for fungistatic activity than is our method. In most cases the sporostatic activity of the compounds was found to be higher than the fungistatic, although the conditions of testing were similar. This might perhaps be attributed to the shorter periods of time required for testing of the former.

Among the phenoxyalkyl derivatives (Table II), the fungistatic and fungicidal activity appears to be higher in the phenoxypropyl than in the phenoxyethyl series. On the other hand, the halogen-substituted phenoxybutyl thiocyanates were generally less active than the corresponding compounds with shorter chain length, whereas the sporostatic activity is more uniformly distributed. Bromine substitutions in the aromatic ring resulted in compounds of higher activity in the phenoxypropyl and phenoxyethyl series. The toloxy groups of the ethyl, propyl, and butyl series, irrespective of the methyl position, were all only weakly active, comparable to the activity of the phenoxybutyl preparations.

With the Schamberg and Kolmer (10) technique, the fungistatic activity, tested with and without 10% rabbit serum, indicates (Fig. 1) the more pronounced effectiveness of γ(*p*-bromo)phenoxypropyl thiocyanate among the preparations reported here. The highest dilutions showing fungistasis, without serum, were: γ(*p*-bromo)phenoxypropyl thiocyanate 1:90,000, undecylenic acid 1:25,000, propionic acid 1:10,000, and 8-hydroxyquinoline benzoate 1:35,000. With the addition of 10% rabbit serum,

TABLE II.—ACTIVITY OF THIOCYANATES

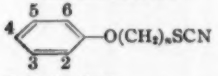
Chemical Structure		Compound	Fungicidal		Fungistatic				Sporostatic	
					Solution		Ointment			
			No. Organ. Tested Against	Average Activity Range (of 25 Tests)	No. Organ. Tested Against	Average Activity Range (of 30 Tests)	No. Organ. Tested Against	Average Activity Range (of 30 Tests)	No. Organ. Tested Against	Average Activity Range (of 10 Tests)
										
Substitutions:										
$n = 3$	Br (4)	β(*p*-Bromo)phenoxyethyl thiocyanate	4	1:100	4	1:50–1:100	3	1:30–1:50	3	1:50,000
	Cl (6)	*o*-Chlorophenoxyethyl thiocyanate	4	1:50	5	1:20–1:50	3	1:20	3	1:20,000–1:50,000
	CH_3 (4)	*p*-Toloxyethyl thiocyanate	4	1:50–1:100	3	1:100–1:200	3	1:50	3	1:20,000–1:100,000
	CH_3 (6)	*o*-Toloxyethyl thiocyanate	4	1:50	3	1:50–1:100	3	1:20	3	1:20,000–1:100,000
	Cl (5)	*m*-Chlorophenoxyethyl thiocyanate	3	1:50	3	1:200	2	1:100	3	1:100,000
	Cl (4)	β(*p*-Chloro)phenoxyethyl thiocyanate	3	1:100	3	1:200	3	1:100	3	1:50,000–1:100,000
	Br (6)	β(*o*-Bromo)phenoxyethyl thiocyanate	3	1:100	4	1:1,000–1:5,000	3	1:50	.	.....
	CH_3 (5)	*m*-Toloxyethyl thiocyanate	4	1:200	3	1:2,000	4	1:100	3	1:20,000–1:200,000
$n = 3$	...	γ-Phenoxypropyl thiocyanate	5	1:50	5	1:1,000	5	1:30–1:50	1	1:20,000
	Cl (5)	γ(*m*-Chloro)phenoxypropyl thiocyanate	4	1:200–1:500	5	1:2,000–1:5,000	3	1:100	3	1:10,000–1:100,000
	Cl (6)	γ(*o*-Chloro)phenoxypropyl thiocyanate	4	1:200–1:1,000	3	1:5,000	3	1:100	3	1:10,000–1:100,000
	Cl (4)	γ(*p*-Chloro)phenoxypropyl thiocyanate	3	1:100	3	1:1,000	2	1:100	3	1:10,000–1:100,000
	Ch_3 (5)	*m*-Toloxypropyl thiocyanate	3	1:100–1:500	4	1:200–1:1,000	3	1:20–1:50	3	1:20,000
	Br (4)	γ(*p*-Bromo)phenoxypropyl thiocyanate	5	1:500–1:1,000	4	1:60,000–1:90,000	4	1:200–1:400	3	1:50,000–1:100,000
$n = 4$	...	δ-Phenoxybutyl thiocyanate	3	1:50–1:100	4	1:100	3	1:30–1:50	3	1:10,000–1:20,000
	CH_3 (4)	*p*-Toloxybutyl thiocyanate	4	1:50	4	1:100	2	1:20	3	1:2,000–1:100,000
	CH_3 (6)	*o*-Toloxybutyl thiocyanate	4	1:50–1:100	4	1:100	2	1:30	3	1:2,000–1:200,000
	Br (4)	δ(*p*-Bromo)phenoxybutyl thiocyanate	5	1:50	4	1:50–1:100	4	1:20	3	1:1,000–1:200,000
	Cl (5)	δ(*m*-Chloro)phenoxybutyl thiocyanate	4	1:100	5	1:100–1:200	3	1:30	3	1:20,000–1:200,000
	Cl (6)	δ(*o*-Chloro)phenoxybutyl thiocyanate	4	1:200–1:500	5	1:500–1:2,000	3	1:30	3	1:20,000

TABLE III.—CONTROLS

Formula	Compound	Fungicidal		Fungistatic				Sporostatic	
		No. Organ. Tested Against	Activity Range	No. Organ. Tested Against	Activity Range	No. Organ. Tested Against	Activity Range	No. Organ. Tested Against	Activity Range
C_3H_5NCS	Allyl isothiocyanate	5	1:100–1:200	3	1:40,000	3	1:100		
C_3H_5NCS	Ethyl isothiocyanate	3	1:50	3	1:10,000	3	1:100		
C_6H_5NCS	Phenyl isothiocyanate	3	1:50	3	1:10,000	3	1:100		
C_2H_5COOH	Propionic acid	4	1:30–1:50	3	1:5,000–1:10,000				
$C_{11}H_{20}O_2$	Undecylenic acid	4	1:40–1:50	3	1:15,000–1:20,000				
$C_7H_6O_2$	Benzoic acid	3	1:10–1:20	3	1:2,000–1:5,000				
C_9H_7ON	8-Hydroxyquinoline	3	1:50	3	1:25,000–1:35,000				
$C_4H_6O_3$	Dehydroacetic acid	3	1:20–1:30	3	1:5,000				
EtOH (95%)	Solvent	5	Inactive	.	.....	.	...	3	1:5
	Zephiran[b]	.	.....	.	.....	.	...	3	1:15,000–1:4,000

[a] Ointment composition: polyethylene glycol 300—80 to 80.9%, "Carbowax" 4000—19%, test compound—0.1 to 1.0%.
[b] Zephiran: a mixture of alkyl radicals (C_8H_{17} to $C_{18}H_{37}$).

Fig. 1.—Fungistatic activity against *T. mentagrophytes*. ▨ with 10% serum, □ without serum. *1*, Undecylenic acid. *2*, 2.5% 8-Hydroxyquinoline benzoate. *3*, Propionic acid. *4*, 1.0% γ(*p*-Bromo)phenoxypropyl thiocyanate.

the results were as follows: γ(*p*-bromo)phenoxypropyl thiocyanate 1:40,000, undecylenic acid 1:5,000, propionic acid 1:1,000, and 8-hydroxyquinoline benzoate 1:9,000.

In a recent paper Oster[1] expressed the opinion that the treatment of dermatophytosis has to date only been attempted with preparations having rather mild fungicidal activity. It is his belief that this has resulted in drug fastness and resistance of causative organisms, with the consequence that there has been a great increase in the chronic cases of tinea pedis, for example. Also there have been very few occasions when extremely effective compounds have been available or prescribed for clinical trials. He also presented clinical evidence in support of his belief in the use of more active drugs to combat dermatophytosis. The results of our *in vitro* study appear to support Oster's view that more active preparations be used. From a highly effective group of fungicides we have chosen the most active —γ(*p*-bromo)phenoxypropyl thiocyanate—for clinical trial against dermatophytosis. This compound has been shown to possess greater activity, against a representative group of pathogenic fungi, than any of the active compounds used today in the popular brands of fungicidal preparations.

CONCLUSIONS

1. A highly effective antifungal thiocyanate has been presented in this report.

2. The order of antifungal activity of γ(*p*-bromo)phenoxypropyl thiocyanate appears to be higher than that of any other available and presently used compound upon comparative testing.

[1] Read before the New York Academy of Science meeting held January 27, 1950.

REFERENCES

(1) Walton, L., Herbold, M., and Lindegren, C., *Food Research*, **1**, 163(1936).
(2) Lovell, T. H., *ibid.*, **2**, 435(1937).
(3) Foter, M. J., and Golick, A. M., *ibid.*, **3**, 609(1938).
(4) Foter, M. J., *ibid.*, **5**, 147(1940).
(5) Walker, J. C., Morell, S., and Foster, H. H., *Am. J. Botany*, **24**, 536(1937).
(6) Hooker, W. J., *et al.*, *ibid.*, **30**, 632(1943).
(7) Davies, W. H., and Sexton, W. A., *Biochem. J.*, **40**, 331(1946).
(8) Burlingame, R. M., and Reddish, G., *J. Lab. Clin. Med.*, **24**, 765(1939).
(9) Golden, M. J., and Oster, K. A., THIS JOURNAL, **36**, 359(1947).
(10) Schamberg, J. F., and Kolmer, J. A., *Arch. Dermatol. and Syphilol.*, **6**, 746(1922).

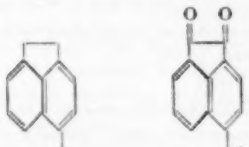
The Fungistatic Properties of Acenaphthene Derivatives*,†

By JAMES E. McDAVID‡ and TROY C. DANIELS§

A group of substituted acenaphthenes and acenaphthenequinones has been prepared and tested, by an agar cup-plate technique, for their fungistatic effect on *Trichophyton mentagrophytes*. Moderate activity was exhibited by several of the compounds.

ANTIFUNGAL activity has been reported by several authors for compounds which contain the acenaphthene nucleus. Bateman and Henningsen (1) have shown that acenaphthene inhibits the growth of *Fomes annosus*, a fungus which infests wood. Inhibition of the growth of *Aspergillus niger* by acenaphthene is reported by Ivanov and Makrinova (2). Ladd (3) has recently described certain antifungal properties of acenaphthenequinone.

In this laboratory a group of acenaphthene and acenaphthenequinone derivatives has been prepared and tested for fungistatic activity. These compounds are denoted by Formulas I and II, in which R is Cl, Br, NO_2, NH_2, SO_3Na, SO_2NH_2, or COOH, and R′ is Cl or Br.



(I) (II)

* Received December 13, 1950, from the College of Pharmacy, University of New Mexico, Albuquerque.

This paper is based on a portion of a thesis submitted to the College of Pharmacy, University of California, by James E. McDavid in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† This work was made possible by the award of a fellowship to J. E. McDavid by the American Foundation for Pharmaceutical Education.

‡ Present address: College of Pharmacy, University of New Mexico, Albuquerque.

§ Dean, University of California, College of Pharmacy.

EXPERIMENTAL[1]

Preparation of Compounds

5-Chloroacenaphthene.—This compound was obtained by the method of Crompton and Walker (4). A sample purified through the picrate consisted of nearly colorless plates, m. p. 69.5 to 70° (uncorr.); reported, 70.5°.

5-Bromoacenaphthene.—Bromination of acenaphthene in warm carbon tetrachloride or acetic acid yielded, after crystallization from ethanol, bright yellow plates, m. p. 51–53° (uncorr.); reported, 51° (4).

5-Nitroacenaphthene.—The nitration of acenaphthene by the method of Quincke (5), as modified by Graebe and Briones (6), yielded yellow needles from ethanol; m. p. 101 to 101.5°; reported, 102° (5), 106° (6, 7). The melting point on repeated crystallization changed in an anomalous manner. After one crystallization of crude material (ligroin-petroleum ether) the melting point was 99–101°. After two subsequent crystallizations (ethanol) the melting point had risen to 105 to 105.5°. After further crystallizations from ethanol, the melting point decreased to the value first mentioned and remained constant.

Anal.—Calcd. for $C_{12}H_9NO_2$: C, 72.36; H, 4.52. Found: C, 72.41; H, 4.47.

5-Aminoacenaphthene.—The nitro derivative was reduced by the method of Fleischer and Schranz (8). The product, after crystallization from toluene-petroleum ether and ethanol-water, consisted of pale tan, fluffy needles, m. p. 105.5°; reported, 108°.

Anal.—Calcd. for $C_{12}H_{11}N$: C, 85.21; H, 6.51. Found: C, 84.71; H, 6.53.

Acenaphthene-5-sodium Sulfonate.—The method of Dziewonski and Stolywho (9) yielded a white microcrystalline product.

Acenaphthene-5-sulfonamide.—Treatment of the sulfonate with PCl_5, followed by ammonium hydroxide, yielded pale tan crystals, m. p. 225–226°; reported, 222–223° (9).

[1] All melting points corrected, unless otherwise specified.

5-Acenaphthenecarboxylic Acid.—5-Acenaphthenylmethyl ketone was prepared by the Friedel-Crafts method, as described by Fieser and Hershberg (10). This compound, m. p. 67 to 68.5° (reported, two forms, one melting at 57 to 57.5°, and the other, 69.5 to 70°), was oxidized by sodium hypochlorite according to the standard technique (11). The product obtained was found to exist in two modifications, one yellow and the other colorless. Both had identical melting properties [m. p., 221–222° with decomposition; reported, 222–223° (10)], and did not differ, within the limits of experimental error, in elementary analysis. Certain other differences in physical properties were noted (12), but it is not pertinent to consider them in this paper.

Anal.—Calcd. for $C_{13}H_{10}O_2$: C, 78.79; H, 5.05. Found: C, 78.80; H, 5.19.

Acenenaphthenequinone.—This compound was prepared by the established method (13), m. p. 258–261°; reported, 261° (14).

5-Chloroacenaphthenequinone.—The oxidation of 5-chloroacenaphthene as described by Dziewonski and Zakrzewska-Barnaowska (15) yielded yellow needles, m. p. 202–203°; reported, 192–193°.

Anal.—Calcd. for $C_{12}H_5ClO_2$: C, 66.67; H, 2.32. Found: C, 65.62; H, 2.33.

5 - Bromoacenaphthenequinone.—5 - Bromoacenaphthene was oxidized according to the directions of Graebe and Guinsbourg (16). Yellow needles, m. p. 224–228°, were obtained; reported, 194° (16), 238° (17).

Anal.—Calcd. for $C_{12}H_5BrO_2$: C, 55.17; H, 1.91. Found: C, 54.71; H, 2.00.

ANTIFUNGAL TESTS

The compounds described above were screened for fungistatic action by a cup-plate method similar to that described by Burlingame and Reddish (18).[1] A well, 12 mm. in diameter, was made in the center of each assay plate, by removing a plug of the Sabouraud agar with a sterile cork borer. The plates were inoculated from seven-day-old cultures of *Trichophyton mentagrophytes*. A mixture of spores and mycelia was transferred to the test plates by gently rolling sterile cotton swabs on the ends of applicator sticks, first over the stock culture, and then over the agar to be seeded.

All the substances tested, except acenaphthene-5-sodium sulfonate, were dissolved in ethanol or ethanol-acetone 7:3. These solvents were shown to have no effect on the growth of the test organism. The sodium sulfonate was dissolved in water. All concentrations were 0.1% of the acenaphthene derivative.

After incubation at 26° for seven days, the fungistatic activity of the compounds placed in the central wells was evaluated in terms of the radius segment[2] of the inhibition zones surrounding the wells. The results represent the average value of two determinations. Table I summarizes the observations.

[1] The screening tests were performed by Mrs. Virginia Pickering, Senior Laboratory Technician.

[2] Radius of the zone of inhibition, minus the radius of the well (6 mm.) made in the agar.

DISCUSSION

Although all the compounds show a certain degree of inhibition, in none of these substances is this inhibition marked. The ability to depress fungus growth shown by this series of acenaphthene derivatives suggests that an inherent property of the acenaphthene nucleus is responsible for the activity. Further chemical investigation might yield compounds which enhance this inherent property and which would display sufficiently strong action to be of interest clinically.

SUMMARY

Eleven compounds which contain the acenaphthene nucleus have been tested for fungistatic activity.

None of the compounds tested show more than moderate activity against *Trichophyton mentagrophytes*.

TABLE I.—FUNGISTATIC ACTIVITY OF CERTAIN ACENAPHTHENE AND ACENAPHTHENEQUINONE DERIVATIVES

Compound	Clear Zone,[a] Mm.
Acenaphthene	4
5-Chloroacenaphthene	5
5-Bromoacenaphthene	3
5-Nitroacenaphthene	3
5-Aminoacenaphthene	5
Acenaphthene-5-sodium sulfonate	2
Acenaphthene-5-sulfonamide	3
5-Acenaphthenecarboxylic acid	10
Acenaphthenequinone	7
5-Chloroacenaphthenequinone	10
5-Bromoacenaphthenequinone	5

[a] Measured as radius segment, as described in text.

REFERENCES

(1) Bateman, E., and Henningsen, C., *Proc. Am. Wood-Preservers Assoc.*, **1923**, p. 136.
(2) Ivanov, N. N., and Makrinova, N. A., *Compt. rend. acad. sci. U. R. S. S.*, **30**, 358(1941).
(3) Ladd, E. C., U. S. pat. 2,435,501, February 3, 1948.
(4) Crompton, H., and Walker, M., *J. Chem. Soc.*, **101**, 959 (1912).
(5) Quincke, F., *Ber.*, **20**, 600(1887).
(6) Graebe, C., and Briones, N., *Ann.*, **327**, 80(1903).
(7) Morgan, G. T., and Stanley, H. M., *J. Soc. Chem. Ind. (London)*, **43**, 343T(1924).
(8) Fleischer, K., and Schranz, K., *Ber.*, **55**, 3253(1922).
(9) Dziewonski, K., and Stolywho, T., *Bull. intern. acad. polon. sci.*, **1924A**, p. 159.
(10) Fieser, L. F., and Hershberg, E. B., *J. Am. Chem. Soc.*, **61**, 1272(1939).
(11) Newman, M. S., and Holmes, H. L., "Organic Syntheses," Vol. II, John Wiley and Sons, New York, 1943, p. 428.
(12) McDavid, J. E., thesis, University of California, 1950.
(13) Maxwell, C. S., and Allen, C. F. H., "Organic Syntheses," **24**, 1(1944).
(14) Graebe, C., and Gfeller, E., *Ber.*, **25**, 652(1892); *Ann.*, **276**, 1(1893).
(15) Dziewonski, K., and Zakrzewska-Barnaowska, M., *Bull. intern. acad. polon. sci.*, **1-2A**, 65(1927).
(16) Graebe, C., and Guinsbourg, M., *Ann.*, **327**, 85 (1903).
(17) Rule, H. G., and Thompson, S. B., *J. Chem. Soc.*, **1937**, p. 1761.
(18) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 765(1939).

Studies on Sex Difference in Resistance to Ouabain in the Albino Rat*,†

By HARALD G. O. HOLCK‡ and KAZUO K. KIMURA§

The presence of sex difference to ouabain, as judged by the average lethal doses (LD_{50}'s) in male and in female rats, has been confirmed and demonstrated. The LD_{50} at different age levels for rats has been determined and is reported. The influence of factors such as methods and frequency of administration, solvent, nature of anesthetic, and castration on sex difference in resistance to ouabain have been intensively studied and the results of these investigations are also reported.

IN THEIR extensive studies on the albino rat, Hatcher and Eggleston (1), Hatcher (2), and Heubner and Nyary (3) administered ouabain apparently without considering sex as a possible source of variation. However, inasmuch as Heubner and Nyary employed urethane anesthesia, sex difference to ouabain probably would not have been significant, since the anesthetic used has been found to be an important factor (4). The first report on higher female resistance to ouabain in the albino rat was made by Holck and Smith (5). The purposes of the present investigation are: (*a*) to confirm the presence of sex difference in resistance to ouabain in the albino rat, (*b*) to determine the average lethal dose (LD_{50}) at different age levels, and (*c*) to study the influence of certain additional factors, such as methods and frequency of administration, solvent, various anesthetics, and castration, on sex difference in resistance to ouabain.

EXPERIMENTAL

Part I: The Effect of Age and Sex on the Average Lethal Dose of Ouabain in the Albino Rat

The rats were bred from Wistar stock in our animal quarters and were kept on a diet of Purina Dog Chow and water *ad lib*. The room temperature was approximately 25°. Before each experiment the rats were fasted overnight. Fresh solutions of ouabain in 0.9% sodium chloride solution were prepared for each experiment, and each rat received a single subcutaneous injection in the abdominal area.

A total of 464 females and 446 males were studied at four age levels, as presented in Table I. As far as possible, litter mates were distributed evenly among the various dosage groups within each sex and age group. Of the 243 rats in the eight- to eleven-month-old group, 128 were eight to nine months old and 115 were ten to eleven months old. Data recorded were identification, age, weight, sex, dose, time of injection, fate, and all necessary comments for each rat.

Our results indicated that the greatest sex difference to ouabain falls in the two-month-old group, in case of which the (LD_{50}) was 60% higher for the females than the males, though about the same variation was noted in case of the four-month-old rats (53% difference). However, in an earlier set of experiments in which data from several days of experimentation were combined, the difference was much greater in case of the four-month-old groups than that for the two-month-old groups, namely about 100% *vs.* about 25%. In the case of the 216 four-month-old rats the LD_{50}'s were 133 *vs.* 66, and in the case of the 212 two-month-old rats they were 137 *vs.* 110 mg./Kg. for the females and males, respectively. In the case of the eleven-month-old rats, the females required only 30% more ouabain than the males for the LD_{50}. Further analysis indicated that the eight- to nine-month-old rats and the ten- to eleven-month-old rats exhibited the same degree of sex variation to ouabain. In the case of the one-month-old rats the difference in the LD_{50}'s was not significant.

Part II: Effects of Varying the Method of Administration, the Dosage Calculation, and the Solvent

Having established that sex difference in resistance to ouabain existed in the albino rat, a series of experiments were conducted to investigate some of the factors which may influence the dosage or the sex difference, or help explain the latter.

The purposes of the first experiment were to ascertain whether an economical quantitative method could be developed in which small doses of ouabain could be injected at five-minute intervals to cause death after a suitable number of doses, and to compare results of subcutaneous with intraperitoneal injections of the drug. The results are presented in Table II. They indicated that the multiple injection technique was practical; in the case of the subcutaneous injections ten sites of injection were used in rotation. Although the average lethal dose was 24% higher for the subcutaneous than for the

* Received December 5, 1950, from the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, Lincoln.

Abstracted in part in *Federation Proc.*, 3, 75(1944). The major portions of this article are from the Master's thesis of K. K. Kimura.

† This project was aided by a grant to the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

‡ The ouabain used in all experiments, except those reported in Tables II, IV, and V, inclusive, was kindly supplied by S. B. Penick and Co. We are indebted to Eli Lilly and Co. for the pentobarbital used. We also wish to thank R. I. Isaksen, E. Kimura, B. W. McCashland, T. S. Miya, C. C. Riedesel, C. A. Stone, L. S. Wu, and Patricia Lear for aid in conducting the tests.

§ Present address: St. Louis University School of Medicine, 1402 South Grand Blvd., St. Louis 4, Mo.

TABLE I.—SUMMARY OF EXPERIMENTS ON SEX DIFFERENCE TO OUABAIN IN THE ALBINO RAT AT VARIOUS AGE LEVELS[a]

Age in Months[b]	One		Two		Four		Eight–Eleven	
Sex	F	M	F	M	F	M	F	M
Total No. of rats	75	87	118	116	108	108	99	99
Dose range in mg./Kg.	50–176	50–176	66.1–176	37.8–133	66.1–202	43.5–133	66.1–153	50–115.7
LD_{50} in mg./Kg. + S. E.	93.7 ± 4.9	100.3 ± 4.8	115.5 ± 3.9	72.0 ± 3.5	132.9 ± 5.3	87.0 ± 3.8	107.0 ± 3.5	82.5 ± 2.3
Ratio of LD_{50}'s (F/M) + S. E.	0.93 ± 0.06		1.60 ± 0.09		1.53 ± 0.09		1.30 ± 0.04	
Duration of tests in days	2		1		1		2	

[a] Each rat was given a single subcutaneous injection of ouabain (1% in isotonic sodium chloride solution). The dose increases were 15%. The LD_{50} represents the dose lethal to 50% of the animals used as calculated by the method of Kärber (6).
[b] One month represents a range of 0.9 to 1.1 months; two months = 1.8 to 2.3 months; and four months = 4.0 to 4.8 months.

intraperitoneal administration, this difference was not statistically significant since the t factor was only 1.7.

With only five-minute intervals, two experimenters can handle relatively few rats in any one test. Therefore, in a second experiment, ten-minute intervals between injections were compared with five-minute intervals, to ascertain if sex variation could be demonstrated equally well with the longer interval between administrations. Holck and Fink (7) had previously observed that rats which were given a small dose of pentobarbital sodium every ninety minutes developed such tolerance that they continued to destroy such small doses every ninety minutes over a number of days. Holck and Duis (8) also had noted that when albino or hooded rats were given very small doses of ouabain at five-minute intervals the females developed tolerance to such a degree that a large number of injections were needed to cause death. When animals developed such tolerance, abnormal amounts of liquid sometimes had to be injected during the course of an extended administration. With ouabain, extra-cardiac actions may then complicate the interpretations. To circumvent these complications, the technique of increasing each successive dose of ouabain by 4.5% was adopted in this experiment, a method previously employed by Holck and Mathieson (9). A summary of the results of this experiment is presented in Table III.

The results indicated that the sex variation may be demonstrated by employing either the five- or ten-minute interval between doses, provided the initial dose was properly chosen. If the initial dose was too small, as seen in the first of the ten-minute interval trials, apparently both sexes developed tolerance; thus, when tolerance to ouabain had developed, the male rats were just as resistant as the females, at least as far as the fatal dose was concerned. However, in case of the males in which tolerance was induced, early signs of poisoning, such as tremors, salivation, and mild convulsions, appeared in about an hour and twenty minutes, or long before the final injection; on the other hand, the females showed such symptoms mainly after several hours, close to the time of death. In either sex, the

TABLE II.—COMPARISON OF SUBCUTANEOUS AND INTRAPERITONEAL INJECTIONS OF OUABAIN[a]

Route of Administration	Av. No. of Mg./Kg.	Standard Error
Intraperitoneal	48.2	4.9
Subcutaneous	59.8	4.9

[a] Six four-month-old male albino rats in each group were given ouabain as 1.2% solution in distilled water. Each rat was given 7.2 mg./Kg. of ouabain every five minutes until death.

TABLE III.—SUBCUTANEOUS INJECTIONS OF OUABAIN AT FIVE- AND AT TEN-MINUTE INTERVALS, STARTING WITH VARIOUS INITIAL DOSES[a]

	Initial Dose, Mg./Kg.	No. of Males	Av. No. of Mg./Kg.	No. of Females	Av. No. of Mg./Kg.	Ratio, F/M	t, M vs. F
			Five-Minute Intervals				
A	5.10	12	95	12	276	2.91	6.50
B		14	113	14	207	1.83	4.35
			Ten-Minute Intervals				
A	5.10	12	200	12	229	1.14	0.87
B		29[b]	259	30[b]	246	0.95	0.90
A	8.64	12	193	12	250	1.29	1.66
B		15	214	15	255	1.19	1.85
A	11.9	12	153	12	224	1.46	3.12
B		15	160	15	251	1.57	3.43
B	20.2	12	121	12	208	1.72	4.83

[a] Groups of four- to four and one-half-month-old albino rats were injected with 1% ouabain in isotonic sodium chloride solution. Within each group the initial dose was increased by 4.5% every five or ten minutes, respectively, and injections continued until death. Sample A was Penick Ouabain, 4910 RLA, and sample B was Penick Ouabain, U. S. P.
[b] Two tests combined.

rats usually died showing marked salivation and marked clonic convulsions. The results also showed that no consistent difference in fatal dosage existed between samples A and B of ouabain, as judged by comparing the fatal doses for each sex separately when starting with the same initial dose.

In our third and fourth sets of experiments the influence of solvent upon the potency of ouabain was studied. Subcutaneous administration was employed in the first phase of this work, in which ouabain was dissolved in either triple distilled water or in 0.9% sodium chloride solution. The results are shown in Table IV.

TABLE IV.—EFFECT OF SOLVENT ON ACUTE MORTALITY IN FOUR- TO FIVE-MONTH-OLD ALBINO RATS WHEN OUABAIN IS ADMINISTERED SUBCUTANEOUSLY[a]

Dose, Mg./Kg.	Distilled Water	Sodium Chloride, 0.9% Solution	Sex
87.5	0/15	7/18	Males
100.6	0/15	8/18	Males
133	2/11	8/11	Males
153	2/13	12/21	Females
176	1/13	14/17	Females

[a] The results represent a combination of data from three experiments carried out at approximately six-month intervals.

The data clearly showed that in either sex the fatalities were very much higher, dose for dose, when ouabain was dissolved in the saline solution than in distilled water. This was similar to what occurs with insulin, in that Trevan and Boock (10), who dissolved the insulin in isotonic sodium chloride solution, reported a much smaller convulsant dose in mice than Marks (11), who employed distilled water as his solvent.

In the second phase of the study, distilled water, isotonic sodium chloride solution, and Locke-Ringer solution were compared as solvents for ouabain given by the intraperitoneal route. The results are presented in Table V.

TABLE V.—EFFECT OF VARIOUS SOLVENTS ON OUABAIN TOXICITY[a]

Solvent	Av. No. of Mg./Kg.	Standard Error
Distilled water	122.8	11.2
0.9% Sodium chloride solution	150.5	10.0
Locke-Ringer solution	103.6	8.9

[a] Female albino rats, nine to twelve months old in groups of 10, were given 6.6 mg./Kg. of ouabain (1% solution) intraperitoneally every five minutes until death. These rats were survivors from low doses of ouabain administered one month previously.

The difference between the number of injections with normal saline solution and the Locke-Ringer solution was highly significant in that the t factor was 3.6. Thus, the additional ions in the latter solvent, especially the calcium ions, apparently increased the toxicity of ouabain. This was similar to the results obtained by Gold and Edwards (12), who reported that there was a marked synergism between calcium and ouabain on the heart. On the other hand, although there was tendency for ouabain to be more toxic in distilled water than in isotonic sodium chloride solution, the difference was probably not significant ($t = 1.82$). The difference between results with Locke-Ringer solution and distilled water as solvents was not significant ($t = 1.3$).

The ratio of heart weight to body weight gradually decreases as rats increase in size (13). Hence, the sex difference to ouabain might largely be due to the fact that adult females, on an average much lighter than adult males, would receive less ouabain per Gm. of heart when the dosage is computed on the basis of body weight. If this explanation were valid, smaller rats of the same age and sex should require a larger fatal dose, and males and females of the same weight should require the same average dose when calculated on the basis of body weight. To investigate this possibility, the 12 heaviest females and the 12 lightest males were selected from a large group of four-month-old rats. The light males and the heavy females were nearly of the same weight. Similar sized groups of the heaviest males and the lightest females are included for the sake of comparison. The results on the comparative resistance of these groups are summarized in Table VI.

TABLE VI.—INFLUENCE OF BODY WEIGHT ON RESISTANCE TO OUABAIN[a]

Sex	Av. Wt., Gm.	Wt. Range, Gm.	Av. No. of Mg./Kg.	S. E.	Range of No. of Injections
M	214	205–227	71.9	12.3	5–28
M	154	142–170	71.3	10.6	6–25
F	151	142–167	109.6	12.5	8–27
F	114	108–121	139.3	13.0	12–34

[a] Ouabain, 6.6 mg./Kg., was administered as a 1.2% solution in distilled water intraperitonealy at five-minute intervals to four-month-old albino rats until death. Eleven rats were used in the heavy female group; all other groups had 12 rats each.

The results showed that the average lethal doses for heavy and light male rats, differing almost 40% in their average weights, were practically identical. Though the difference between the LD_{50}'s of heavy and light females, differing about 32% in their average body weights, was 27%, this difference was not statistically significant ($t = 1.65$). On the other hand, a comparison of the results with males and females of almost equal weight showed a difference which was significant ($t = 2.34$). A comparison of the results with heavy males and light females indicated the existence of a significant sex difference to ouabain ($t = 3.76$). Thus, results obtained by this technique indicated that variation to ouabain in the albino rat cannot be explained on the basis of difference in body weight between the two sexes.

To study this problem further, a different method of investigation was employed. In this case the dosage of ouabain was computed on the basis of heart weight instead of body weight. Heart weights were estimated from the body weights according to Table 145 in the memoir by Donaldson (13). The rats were anesthetized with pentobarbital sodium adjusted according to sex (14), so as to enable one person to conduct the entire experiment. The results are summarized in Table VII.

TABLE VII.—ADMINISTRATION OF OUABAIN ON THE BASIS OF HEART WEIGHT[a]

Sex	Av. Wt., Gm.	Av. Estimated Heart Wt., Mg.	Av. Actual Heart Wt., Mg.	Av. No. of Mg. of Ouabain per Gm. of Actual Heart Wt. and S. E.
M	290	1,072	1,013	14.4 ± 1.9
F	172	714	611	42.6 ± 6.0

[a] Nineteen six- to seven-month-old albino rats (nine males and ten females) were given 1.6 mg. of ouabain in distilled water (1.2%) intraperitoneally per estimated Gm. of heart weight every five minutes until death. The males and females were injected subcutaneously with 30 and 20 mg./Kg. of pentobarbital sodium, respectively, forty minutes prior to ouabain injections.

The average estimated heart weight for the females was about 17% greater than the actual weight, while it was only about 6% greater with the male rats. Thus, the males received less ouabain per Gm. of heart than the females. Nevertheless, the males died with approximately one-third of the dose that was fatal to the females. A comparison of the average fatal doses per Gm. of heart weight gave a t factor of 4.47, indicating a significant difference. The t factor would have been even greater had the heart weights been estimated more accurately. The results thus confirmed the conclusion drawn from the previous experiment, namely, that relative proportion of the heart to body weight did not explain the sex difference to ouabain.

Part III: Effects of Choice of Anesthetic and of Castration

In further tests to elucidate the mechanism of action and the factors involved in the sex difference to ouabain, the effects of castration of both sexes were investigated. This was in part due to the fact that Holck, *et al.* (14), reported that castration of the male rat lengthened the response to certain short-acting barbiturates to which the males were more resistant. In the first of the present experiments, castration of both sexes was carried out about two and one-half months prior to ouabain medication. The results are presented in Table VIII.

These results indicated that castration of either sex did not significantly alter the response to ouabain. Whether spaying had any influence upon the relation of dosage to body weight was not ascertained. In previous studies by others (15–18) on this problem, employing rats spayed at twenty-four to twenty-six days, rather than at two months, the age employed in our rats, complex results were reported. Apparently, at the age of seventy days, the relation in spayed and control rats was the same; at three months, the ratio of heart weight to body weight was increased; at six months it showed no significant difference; and at about twelve months, the ratio was decreased in the spayed rats as compared with the controls. However, in view of the results of our experiments upon the relation of heart weight to body weight and of the age of our rats (four and one-half to six months), it seems unlikely that the increased body weight after spaying was a major factor. The difference between the normal females and males was significant, as indicated by a t value of 3.8. The difference between normal females and castrated males ($t = 2.5$), and between normal males and spayed females ($t = 2.7$) likewise were significant. On the other hand, the difference between castrated males and spayed females was not significant ($t = 1.6$). Also, castration of the males did not significantly increase the resistance ($t = 1.15$).

In the hope of securing more uniform results by administering ouabain more directly to the heart, intravenous administration via a blunted 1-in. 19-gauge hypodermic needle inserted and tied into the exposed right external jugular vein was tried. To avoid undue coagulation of blood in the injection needle, injections were made every three minutes. In view of the absence of reports of sex difference (see introduction) to ouabain in the rat when urethane was employed as the anesthetic, this drug was used to induce general anesthesia. The rats of both sexes were castrated under ether anesthesia two months prior to ouabain medication; in all cases the control rats were subjected to a similar operative technique, with the exception that the sex organs were left intact. This experiment was completed in seventeen days and the rats were properly randomized by the use of Latin squares. The solvent was isotonic sodium chloride solution, rather than distilled water. The results are summarized in Table IX.

These results showed that the variation in the number of injections required to kill the rats was again very great, as shown in the "Range of No. of Injections" column; consequently, the standard errors were very large. Simple inspection indicated that castration had no influence upon the fatal dose in either sex, similar to the finding in the previous experiment. It was also shown that with urethane anesthesia no sex difference between normal females and males was present ($t = 1.38$). The absence of such difference may explain why Heubner and Nyary (3), who also employed urethane anesthesia, did not observe any sex difference to digitalis or similarly acting substances.

Having ascertained that urethane, which induced satisfactory anesthesia without undue respiratory

TABLE VIII.—EFFECTS OF CASTRATION ON THE RESPONSE OF THE ALBINO RAT TO OUABAIN[a]

Group	Sex	Av. Wt., Gm.	Av. No. of Mg./Kg.	S. E.	Range of No. of Injections
Normal	M	241	89.1	21.6	7–40
Castrated	M	231	124.7	22.4	4–35
Normal	F	147	200.0	19.4	18–46
Spayed	F	198	175.6	23.2	11–41

[a] The rats were four and one-half months old and were injected with 6.6 mg./Kg. of ouabain (1% solution) every five minutes, intraperitoneally, until death occurred. Each group consisted of ten rats. The solvent was isotonic sodium chloride solution.

TABLE IX.—EFFECTS OF URETHANE ANESTHESIA AND OF CASTRATION ON THE RESPONSE OF THE ALBINO RAT TO OUABAIN[a]

Group	Sex	Av. Wt., Gm.	Av. No. of Mg./Kg.	S. E.	Range of No. of Injections
Normal	M	266	32.5	6.8	3–29
Castrated	M	233	39.5	6.4	4–23
Normal	F	169	47.3	8.8	5–39
Spayed	F	213	41.8	2.8	11–21

[a] The rats were five to six months old, and injected intravenously with 2.5 mg./Kg. of ouabain every three minutes, using 0.5% solution in isotonic sodium chloride solution. Urethane, 1 Gm./Kg., was given intraperitoneally (25% solution). Each group consisted of ten rats.

depression, was not suitable for studies on sex difference to ouabain, pentobarbital sodium was tried again. Since sex difference in resistance to pentobartital existed in the rat, pharmacologically equivalent doses were administered, namely 35 mg./Kg for the males and 22–25 mg./Kg. for the females, using the intraperitoneal route of administration. Castration again was carried out two months before the main experiment, and isotonic sodium chloride solution used as the solvent. The intravenous technique also was the same and total tests were concluded within three weeks. The results are given in Table X.

With pentobarbital anesthesia the results differed in several important aspects. In agreement with the findings in unanesthetized rats, higher female resistance to ouabain was plainly evident ($t = 3.6$). Although the number of injections required for the normal males was less than for the castrates, the difference was not statistically significant ($t = 1.5$). As seen by simple inspection, the results with castrated males and with spayed females were practically identical. A wide range in the number of injections again was seen. It seems of interest to note that in rats receiving ouabain at three-minute intervals, especially after tolerance was built up, the heart beats became very irregular for one and one-half to two minutes after each injection, as ascertained with the stethoscope, but recovered before the next injection. Looking at the results of the several castration experiments, it should be emphasized that in the normal rats and in the pentobarbitalized rats, in which the usual higher female resistance to ouabain was shown, no significant sex difference was present after castration of both sexes.

DISCUSSION

We have shown that a markedly higher resistance to ouabain was present in the adult female as compared with the adult male albino rat. This difference, as seen by a much higher average fatal dose, was absent in the very young rats, but was demonstrated in animals as young as two months and persisted at least to the age of eleven months, which was the maximum age of rats in our studies. The sex difference may also be observed by injecting a small dose of ouabain subcutaneously or intraperitoneally at five- or ten-minute intervals; however, the conditions must be carefully controlled. If the initial dose were too small, or if the time interval between injections were too long, male rats developed tolerance to such an extent that the average fatal doses for the two sexes tended to become equal. The abolition of sex difference, once tolerance has been induced, supported, in principle, the results which Holck and Mathieson (9) obtained with pentobarbital.

Another factor which may greatly influence the action of ouabain was the solvent. Ouabain, when administered subcutaneously, was apparently absorbed much more rapidly from a saline solution than from distilled water, as indicated by a much lower fatal dose. This was not clearly shown when the intraperitoneal method of administration was employed. However, in the latter case, the potency of ouabain in Locke-Ringer solution was markedly greater than in isotonic sodium chloride solution. Inasmuch as no experimentation was carried out on isotonic sodium chloride solution with Ca^{++} and K^{+} added separately, neither their relative influence on the ouabain potency, nor the influence of varying their proportions have been ascertained. Calcium ions alone have repeatedly been reported to be synergistic with ouabain (12). Ouabain dissolved in Locke-Ringer solution was not significantly more potent than ouabain dissolved in distilled water; we have no explanation for this difference.

Inasmuch as the heart weight in proportion to body weight was greater in the females, which were smaller than the males, ouabain effectiveness was compared in female and male rats of the same size and in the two sexes by administering ouabain according to estimated heart weight. In either case, marked sex difference was shown. One explanation for this may be that the female heart *per se* was more resistant to ouabain than that of the male. It was of great interest here that Wollenberger and Karsh (19) have just reported that when the adult, isolated, female rat heart was studied by the closed perfusion method, using a Langendorff preparation, it required 50% more ouabain to stop it than to arrest the isolated heart of the male rat. Such variation was absent in hearts from three-week-old rats.

It is also possible that a part of the mechanism of the sex variation was due to a more rapid excretion of ouabain in the bile by the female rat. Farah has reported that the main route of elimination of ouabain in the rat was by the hepatic route (20).

The effects of castration of both sexes was studied by administering ouabain intravenously every three minutes under pentobarbital and under urethane anesthesia, as well as by the intraperitoneal route every five minutes in unanesthetized animals. Neither when using normal rats nor those anesthetized with urethane, did castration significantly alter the ouabain fatal dose. In fact, under urethane anesthesia it was impossible to demonstrate a significant sex difference between noncastrated

TABLE X.—EFFECTS OF PENTOBARBITAL ANESTHESIA AND OF CASTRATION ON THE RESPONSE OF THE ALBINO RAT TO OUABAIN[a]

Group	Sex	Av. Wt., Gm.	Av. No. of Mg./Kg.	S. E.	Range of No. of Injections
Normal	M	242	21.0	1.7	6–13
Castrated	M	244	32.8	7.5	5–43
Normal	F	161	83.0	16.7	11–74
Spayed	F	207	31.5	4.5	6–27

[a] The rats were injected intravenously with 2.5 mg./Kg. of ouabain every three minutes, using 0.5% solution in isotonic sodium chloride solution. Each group consisted of ten six- to seven-month-old rats.

males and females. However, in case of pentobarbitalized rats the usual higher female resistance was plainly seen and spaying definitely decreased the number of injections required to cause death. In no case was there a significant difference in fatal dosage in the two castrate groups.

Our results with the various multiple-dose administrations indicate one further thing, namely that the rat seemed entirely unsuited as a test animal in the bioassay of ouabain. The results in one case where two samples were compared were not consistent; one cannot be certain of the extent to which the convulsant action became a factor in the toxicity of ouabain upon the heart. This action was especially likely to occur if one administered a number of small doses, whereas with a single large dose the rat died from cardiac failure without convulsions as reported by Gold, *et al.* (21). Another serious difficulty was the fact that the range in doses necessary to kill a rat was generally very wide by this method of repeated administrations, so that often the standard error with 12 rats became 15 to 20% of the average.

SUMMARY

1. Sex difference to ouabain in the albino rat was absent at the age of one month, marked at the two- and four-months age levels, and on the decline at the age of eight to eleven months, as judged by the average lethal doses (LD_{50}'s) in the two sexes.

2. Sex difference could also be demonstrated by injecting a small dose every five or ten minutes, provided the initial dose was properly chosen. If it were too small, tolerance developed in both sexes and no sex variation was found.

3. With the subcutaneous method of administration ouabain was more toxic when dissolved in isotonic sodium chloride solution than in distilled water. When administered intraperitoneally, ouabain was more potent in Locke-Ringer solution than in isotonic sodium chloride solution, but not in distilled water.

4. When ouabain was administered by the multiple-dose method to male and female rats of the same age and size, to rule out the possible effect of the proportionally larger female heart, sex difference still was plainly evident. When ouabain was administered by vein in dosages based upon estimated heart weight under pentobarbital anesthesia, sex difference still was obtained.

5. Castration of either sex did not significantly alter the fatal dose of ouabain when administered intraperitoneally every five minutes to unanesthetized rats or intravenously every three minutes to rats under urethane anesthesia. However, under pentobarbital anesthesia, spaying significantly decreased the fatal dose of ouabain when the latter was given by vein every three minutes. In no case was there a significant difference between castrated males and spayed females. Also, with urethane anesthesia, there was no significant sex difference in the response to ouabain in the noncastrated rats.

6. Because of the ease with which some of the rats, especially the females, develop tolerance, there is great variation in the number of doses required to kill rats in a group. This fact, together with the complication of the marked central convulsant action of ouabain, makes the rat unsuitable for the bioassay of ouabain.

REFERENCES

(1) Hatcher, R. A., and Eggleston, C., *J. Pharmacol. Expll. Therap.*, **12**, 405(1919).
(2) Hatcher, R. A., *J. Am. Med. Assoc.*, **61**, 386(1913).
(3) Heubner, W., and Nyary, V., *Arch. expll. Path. Pharmakol.*, **177**, 60(1935).
(4) Kimura, K. K., "Studies on the Influence of Sex and Age upon Resistance to Ouabain in the Albino Rat," Master's thesis, University of Nebraska, 1944.
(5) Holck, H. G. O., and Smith., E. L., *Am. J. Physiol.*, **123**, 104(1938).
(6) Kärber, G., *Arch. expll. Path. Pharmakol.*, **162**, 480 (1931).
(7) Holck, H. G. O., and Fink, L. D., THIS JOURNAL, **29**, 475(1940).
(8) Holck, H. G. O., and Duis, B., unpublished data.
(9) Holck, H. G. O., and Mathieson, D. R., THIS JOURNAL, **33**, 174(1944).
(10) Trevan, J. W., and Boock, E., League of Nations Report on Insulin Standardization, C. H. 398.
(11) Marks, H. P., *ibid.*
(12) Gold, H., and Edwards, D. J., *Am. Heart J.*, **3**, 45 (1927–1928).
(13) Donaldson, H. H., "The Rat," Memoirs of the Wistar Institute of Anatomy and Biology, 2nd ed., 1924, p. 234.
(14) Holck, H. G. O., Kanan, M. A., Mills, L. M., and Smith, E. L., *J. Pharmacol. Expll. Therap.*, **60**, 323(1937).
(15) Freudenberger, C. B., and Hashimoto, E. I., *Am. J. Anat.*, **62**, 93(1937–1938).
(16) Freudenberger, C. B., and Howard, P. M., *Proc. Soc. Expll. Biol. Med.*, **36**, 144(1937).
(17) Freudenberger, C. B., and Billeter, O. A., *Endocrinology*, **19**, 347(1935).
(18) Billeter, O. A., *Am. J. Anat.*, **60**, 367(1946–1947).
(19) Wollenberger, A. W., and Karah, M., personal communication, July, 1950.
(20) Farah, A., *J. Pharmacol. Expll. Therap.*, **86**, 101 (1946).
(21) Gold, H., *et al.*, *ibid.*, **91**, 15(1947).

Some Pharmacologic Actions of 53 Derivatives of Thiazole with Substituents in the 2- and 4-Positions*

By BRADFORD N. CRAVER, WALTER BARRETT, ANNE CAMERON, and JEAN FEICKERT†

In this paper the general pharmacological actions of a series of 53 substituted thiazoles have been summarized in tables. The substituents were either in the 2- or the 4-position, or in both. No substituents were found that would entirely inhibit the inherent toxic effect of the thiazole nucleus. Some members of the series possessed moderate antihistaminic action from which generalizations between structure and this activity could be inferred. These generalizations confirmed principles in connection with other series of antihistaminic compounds commonly recognized as valid.

THE chemical preparation of the 53 thiazole derivatives discussed in the present paper have already been reported (1). Most of the derivatives have substituents in both the 2- and 4-positions, but a minority of the group have substituents in only one of these two positions. This report describes the results of the pharmacological screening of these compounds for activity of potential therapeutic interest.

EXPERIMENTAL

Methods.—All of the methods employed in this study have been previously described (2).

Results.—The results have been summarized in Tables I to IV.

Discussion.—A considerable number of derivatives of thiazole have been studied physiologically since the initial synthesis of this heterocyclic nucleus over seventy years ago (3). Johnson and his associates synthesized a large group of thiazole derivatives with some structurally related to the sympathomimetic amines, whose activities many of their compounds were found to resemble (4). Many of their derivatives also exhibited a local anesthetic action. More recently other derivatives of this heterocyclic nucleus have been shown to possess analgesic activity (5, 6), spasmolytic and analeptic properties (7), histaminic action (8), and, finally, at least two have been studied for a specific goitrogenic activity (9). The last activity was originally reported by French workers (10–12). This led to the clinical trial of 2-aminothiazole in thyrotoxicosis. The side effects of therapy, however, were serious, and in seven of the 129 patients studied by Perrault and Bovet, required withdrawal of the drug. None of the members possessing other actions have found a permanent place in therapy, save the thiazole derivative of sulfanilamide. Certain sulfur derivatives of thiazole have also been shown to exhibit antibacterial action, which led Gibbs and Robinson (13) to attribute it to competition with aneurine because of the thiazole group in vitamin B_1. They showed, however, that the added vitamin failed to influence their bacteriological activity. The failure of any of these derivatives to find a therapeutic application would suggest a toxic effect inseparable from the thiazole group.

A survey of the series of thiazoles described in this paper supports this impression that the thiazole nucleus exerts a toxic action that a wide variety of substituents cannot entirely suppress. The results afford no discerned clue as to the focus of this action. The hypotensive action of many of the compounds in the anesthetized dog was roughly correlative with the inhibitory action of the compounds on the isolated cat heart.

Although none of the compounds exerted a significant antihistaminic or anticholinergic activity, the variation in this action with structure within the series would appear to permit certain generalizations in confirmation of those already accepted concerning antihistaminic activity: (*a*) The compounds with antihistaminic activity also exhibited some degree of anticholinergic activity. This would appear to be additional indirect evidence for the close physiological connection between histamine and acetylcholine, a connection still obscure. (*b*) Antihistaminic activity was extremely weak or absent in those compounds with no substituents in the 2-position (Su-361, Su-362, Su-366, Su-385, Su-403, and Su-494). (*c*) A chlorine atom in the *para*-position of the phenyl ring enhanced the antihistaminic action (Su-739 > Su-682; Su-738 > Su-665). A chlorine atom in the *meta*-position appeared ineffective (*cf.* Su-674 and Su-661 with Su-675 and Su-682, respectively). This confirms the findings of Vaughan and his associates (14). (*d*) An alkyl substituent in the *ortho*-position of the phenyl ring appeared also to have enhanced either the antihistaminic action (Su-668 > Su-665) or the anticholinergic action (Su-678 > Su-675), or both (Su-683 > Su-682). (*e*) Many of the series exhibited a significant oxytocic activity *in vitro*. *In vivo*, they were less effective as oxytocics, and the doses required to stimulate the uterus often induced untoward changes in the blood pressure and respiration.

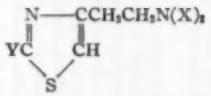
SUMMARY

The general pharmacological actions of a series of 53 thiazoles substituted in the 2- or 4-position or in both positions have been summarized in charts. The thiazole nucleus would appear to

* Received October 13, 1950, from the Research Department, Division of Macrobiology, Ciba Pharmaceutical Products, Inc., Summit, N.J.

† With the technical assistance of James Smith.

TABLE I

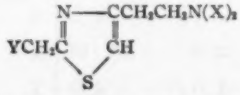


Prepn. No.	Chemical Name "X"	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	G.-P. Ileum (1) Effect	G.-P. Ileum vs. Hist. (2) Dose	G.-P. Ileum vs. Hist. (2) Effect	G.-P. Ileum vs. Asch. (3) Dose	G.-P. Ileum vs. Asch. (3) Effect	Ileum (1)(5) Dose	Ileum (1)(5) Effect	Uterus (1)(5) Dose	Uterus (1)(5) Effect	Perfused Feline Heart (4) Dose	Perfused Feline Heart (4) Effect	Dog (Doses in Mg./Kg I. V.) Dose	Effect on B. P.	Effect on Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-368	Methyl	H	...	...	...	..	...	..	....	..	...	..	100	(I)	1	(S)	S	1: in cat I. V. gave hypotension but had no effect on the uterus.
															2–4.5	(S)	S	
															10	S!I	!S	
Su-366	Ethyl	H	100	0	100	P	100	P	100	0	100	0?	5	(I)	5	I	!S	
													Extra systoles		C. N. S.: S?			
Su-440	Ethyl	Allylamino	100	0	100	0	100	0	10–100	!S	1–10	S	50–100	mI	1	(I)	..	
													Rate and flow Decr.		5–10	!Ipp	(I)	
															C. N. S.: S?			
Su-454	Ethyl	Phenylamino	1–10	(S)	100	P	100	P	100	(I)?	1–10	!S	5	(I)	0.2	0	I	
													20	!I	1	mI	!I	
Su-507	*n*-Propyl	Phenylamino	1–10	(S)	100	P	100	0	10	(S)	1	0	....	...	5–10	0	Var.	3: no effect in dog with T. V. loop.
			100	I	...	..	...	..	....	..	10	!S	50–500	(S)	C. N. S.: S; Epi–			3–5: uterine stimulation of cat *in vivo* but little effect B. P.
Su-450	*n*-Propyl	Allylamino	1–10	(S)	10–100	P	100	P	100	(I)?	1–10	!S	200	(I)	5	0	(S)	5: stimulated cat uterus *in vivo* but had no effect on B. P.
			100	I	...	..	...	..	....	..	...	..	Rate Decr.		10	mIp	..	
															C. N. S.: S; p. pr.–			
Su-453	*n*-Propyl	Phenylamino	1–10	S	10	P	10	P	100	I	1–100	S	50	mI	1	(I)	0	
			100	I	100	I	100	I	....	..	...	..	Rate Decr.		2.5	mIpp	(S)	
															10	Ip	!S	
															Almost lethal			
Su-483	*n*-Propyl	Benzylamino	1–100	(S)	1–10	P	1–10	P	1–10	S	1	S	50	I	1	0	!I	
					100	I	100	I	....	..	10	!S	....	...	..	...	..	
Su-494	Isopropyl	H	1–100	0	10–100	P	1–100	P	10–100	(I)	1–10	(S)?	100–400	(S)	5	(I)	S	
											100	!S	....	...	10	Var.	S	
															C. N. S.: S?			
Su-497	Isopropyl	Allylamino	1–10	(I)	100	P	10–100	P	100	mS	1	0	100	(I)	5	mI	(S)	
			100	0	...	..	...	..	....	..	10	!S	....	...	10	mI	(S)	
															Rate Decr.; p. pr. Incr.; C. N. S.: S?			
Su-477	Isopropyl	Phenylamino	100	0	10	P	10	P	10	(S)	1	(S)	20–50	(S)?	5	(I)	S	
					100	I	100	I	100	(I)	10	!S	100	!Ipp	10	!Ip	!Ip	
													Rate Decr.		Cardiac Irreg.			

TABLE I (*Continued*)

Prepn. No.	Chemical Name "X"	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	G.-P. Ileum (1) Effect	G.-P. Ileum vs. Hist. (2) Dose	G.-P. Ileum vs. Hist. (2) Effect	G.-P. Ileum vs. Ach. (3) Dose	G.-P. Ileum vs. Ach. (3) Effect	Ileum (1)(5) Dose	Ileum (1)(5) Effect	Uterus (1)(5) Dose	Uterus (1)(5) Effect	Perfused Feline Heart (4) Dose	Perfused Feline Heart (4) Effect	Dog (Doses in Mg./Kg. I. V.) Dose	Dog Effect on B. P.	Dog Effect on Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-493	Butyl	Allylamino	100	(S)	1	P	1–10	P	100	(S)	1–10	(S)	20	0	5–10	(I)S	Apnea	
					10	I	100	I	....	..	...	..	50	I	..	...	..	
Su-385	Benzyl	H	...	...	10	P	10	P	10	S	10–100	S	50	(I)?	10	(S)	..	1–10: B. P. Incr. in cat with no effect on uterus *in vivo*.
					100	I	100	I	100	I	...	..	....	...	..	...	..	2: no effect on dog with T. V. loop.
Su-403	Benzyl, ethyl	H	1–100	S	10–100	P	10–100	P	10	(S)	1	S	10–40	I	1–5	(I)	(S)	5–10: stimulation of cat uterus *in vivo* with variable effects on B. P. LD_{50} I. V. rats, 35.
									100–200	S	10	!S	....	...	10	(I)pp	(S)pp	
															Rate Decr.; Epi.−?			

TABLE II

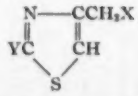


Prepn. No.	Chemical Name "X"	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	G.-P. Ileum (1) Effect	G.-P. Ileum vs. Hist. (2) Dose	G.-P. Ileum vs. Hist. (2) Effect	G.-P. Ileum vs. Ach. (3) Dose	G.-P. Ileum vs. Ach. (3) Effect	Ileum (1)(5) Dose	Ileum (1)(5) Effect	Uterus (1)(5) Dose	Uterus (1)(5) Effect	Perfused Feline Heart (4) Dose	Perfused Feline Heart (4) Effect	Dog (Doses in Mg./Kg. I. V.) Dose	Dog Effect on B. P.	Dog Effect on Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-675	Ethyl	Phenoxy	100	I	10	P	10–100	P	10	I	1	(S)	50	(I)pp	5–10	(I)	0	125: I. P. rats gave convulsions, paralysis and tail erection.
					100	I	....	..	G. P.	..	10	S	Rate Decr.					
											G. P.							
Su-676	Ethyl	*p*-Methyl-phenoxy	10–100	I	10	P	10	P	10	I	10	S	100	mI	5	(I)	(S)	200: I. P. rats gave convulsions, paralysis.
					100	I	100	I	G. P.	..	G. P.	..	Rate Decr.		10	(S)	(S)	
															Epi.+			
Su-678	Ethyl	*o*-Isopropyl-phenoxy	1–100	I	10	P	1	P	10	I	10	S	50	Ip	10	(I)	S	
					100	I	10	I	G. P.	..	G. P.	..	Rate and flow Decr.		Rate Decr.; p. pr.+			
Su-680	Ethyl	*p*-Diphenyl-oxy	10–100	I	10	P	10	P	10	(I)	100	S	50	(I)	5	(S)	0	
					100	I	....	..	G. P.	..	G. P.	..	Rate Incr.		10	IS	S	
															Rate Decr.; S.: S	C. N.		
Su-674	Ethyl	*m*-Chlorophen-oxy	1–10	I	10	P	10	I	10–100	I	100	S?	50	(I)	5	(S)	0	
					100	I	....	..	G. P.	..	G. P.	..	Rate Decr.		10	Var.	(S)	
															Rate Decr.			

TABLE II (*Continued*)

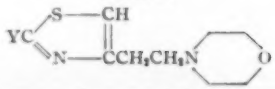
Prepn. No.	Chemical Name "X"	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	Ef-fect	G.-P. Ileum vs. Hist. (2) Dose	Ef-fect	G.-P. Ileum vs. Ach. (3) Dose	Ef-fect	Ileum (1)(5) Dose	Ef-fect	Uterus (1)(5) Dose	Ef-fect	Perfused Feline Heart (4) Dose	Effect	Dog (Doses in Mg./Kg. I. V.) Effect on B. P.	Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-677	Ethyl	2,5-Dimethyl-phenoxy	100	0	10	P	10	P	10	I	10	S	100	(I)	5 (I)	(S)	100: I. P. rats gave convulsions, salivation.
					100	I	100	I	G. P.	..	G. P.	..	....	...	6.9 (I)	S	
Su-686	Ethyl	Thymoxy	100	I	10	P	10	I	100	(I)	100	S	20	(I)	1 (I)p	(I)	
					100	I	...	..	G. P.	..	G. P.	..	....	...	Rate Decr.		
															3.3 !Ip	I	
Su-660	Ethyl	N-Benzyl-N-phenylamino	10	(I)	10	I	10	I	100	0	100	S	200	!Ip	Lethal		
													Flow Decr.; Irreg.		5 (I)Sp	0	
															10 ISp	0	
Su-662	Ethyl	*p*-Methyl-phenylthio	1–100	I	10	P	10	P	100	I	10	S	100	mIpp	P. pr.+; Epi.+		
					100	I	100	I	G. P.	..	G. P.	..	Rate and flow Decr.		5 I	0	
															8.2 Ipp	(S)	
Su-682	*n*-Propyl	Phenoxy	1–10	S	10	P	10	P	10	(I)	10	S	100	(I)?	Rate Decr.; Epi.+		
			100	I	100	I	100	I	G. P.	..	G. P.	..	....	...	1 (I)	0	75: I. P. rats gave convulsions, ataxia and hypnosis.
															5 Ipp	0	
															7.7 Ip	(S)	
Su-679	*n*-Propyl	*p*-Methylphen-oxy	10–100	I	10	P	10	P	10	I	1–10	S	100	(I)?	5 (S)	(S)	100: I. P. rats gave convulsions, salivation, tail erection, paralysis.
															Rate Decr.		
					100	I	100	I	G. P.	..	G. P.	..	Rate Decr.		10 (I)	(S)	
															Rate Decr.; Epi.+		
Su-683	*n*-Propyl	*o*-Isopropyl phenoxy	1	S	1	P	1	P	10	(I)	10	S	50–100	mS	5 I	(S)	
															Rate Decr.; Irreg.		
			10	I	10	I	10	I	G. P.	..	G. P.	..	....	...	10 Ipp	S	
Su-681	*n*-Propyl	*p*-Diphenyloxy	...	...	...	..	...	..	....	..	...	..	50	(I)	5 mI	0	
															10 mIp	0	
Su-661	*n*-Propyl	*m*-Chlorophen-oxy	10	(S)	10	P	1	P	100	I	10	(I)	100	(I)	1 (I)	0	
			100	I	100	I	10	I	....	..	...	..	....	...	4.9 !I	S	
															Epi.+		
Su-685	*n*-Propyl	Thymoxy	10	(S)	1	P	1	P	10	I	10	S	100	(S)	1 (I)	0	
					10	I	10	I	G. P.	..	G. P.	..	....	...	5–10 !I	S	
															Irreg.		
Su-689	*n*-Propyl	2,5-Dimethyl-phenoxy	...	...	...	..	...	..	....	..	...	..	100	0	5 mI	(S)	
													Rate and flow Decr.; Irreg.		9.3 !Ip	S	
															Rate Decr.		
Su-739	*n*-Propyl	*p*-Chlorophen-oxy	10	I	1	P	1	P	10	I	100	S	50	(I)	5 (I)pp	0	
					10	I	10	I	G. P.	..	G. P.	..	Flow Decr.		7.7 mIp	(S)	
Su-807	Butyl	*p*-Chlorophen-oxy	10	I	1	P	1	P	10	I	100	0	50	0	5–10 mI	Sp	100: I. P. rats gave hypnosis, clonic convulsions, ataxia, tail erection.
					10	I	10	I	G. P.	..	G. P.	..	....	...	Rate Decr.		

TABLE III



Prepn. No.	Chemical Name "X"	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	Effect	G.-P. Ileum vs. Hist. (2) Dose	Effect	G.-P. Ileum vs. Ach. (3) Dose	Effect	Ileum (1)(5) Dose	Effect	Uterus (1)(5) Dose	Effect	Perfused Feline Heart (4) Dose	Effect	Dog (Doses in Mg./Kg. I. V.) Effect on Dose	B. P.	Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-462	Piperidino	Phenylamino	100	0	10	P	10	P	100	0	1–10	!S	50	(I)	0.2–5	mI	!S	2–10: no effect on blood pressure or uterus of cat *in vivo*; delayed death.
					100	I	100	I	....	..	...	..	....	...	10	I	Apnea	
Su-386	Piperidino	Benzylamino	100	0	100	P	100	P	100	mI	1	S	50	(I)	1–5	mI	..	5: moderate hypotension in cat with no effect on uterus *in vivo*.
											10	!S	....	...	9	!Ipp	..	
Su-361	Piperidinomethyl	H	1	S	100	0	100	0	100	IS	10	S	50	Asystole	1	(S)	S	0.1: intracarotidly in cat gave moderate hypertension but no salivation.
			10	!S	...	..	...	..	....	..	...	..	200 after atropine	(S)	5	SmIp	..	
															10	Death	..	
Su-737	Piperidinomethyl	*p*-Chlorphenoxymethyl	100	I	10	P	10	P	1–10	I	1–10	S	50	I	5–8.9	(I)	(S)	
					100	I	100	I	G. P.	..	G. P.	..	Flow Decr.		Rate Decr.			
Su-445	Piperidinomethyl	Phenylamino	1–10	(I)	10	P	10	P	100	0	5–100	!S	20	(S)	1	(I)	..	2: no effect on B. P. or uterus of cat *in vivo*.
					100	I	100	I	....	..	...	..	50	!I	5	mIpp	..	
															10	!Ipp	..	
															Rate Decr.; Irreg.			
Su-356	Piperidinomethyl	Benzylamino	10	S	10	P	100	P	100	S	10	!S	5	!I	0.1	S	..	1–5: prolonged hypotension with slight stimulation of cat uterus *in vivo*.
			100	I	100	I	...	..	....	..	...	..	....	...	1–5	I	..	
															Irreg.			

TABLE IV



Prepn. No.	Chemical Name "Y"	Isolated Tissues (All Concentrations, μg./Cc.) G.-P. Ileum (1) Dose	Effect	G.-P. Ileum vs. Hist. (2) Dose	Effect	G.-P. Ileum vs. Ach. (3) Dose	Effect	Ileum (1)(5) Dose	Effect	Uterus (1)(5) Dose	Effect	Perfused Feline Heart (4) Dose	Effect	Dog (Doses in Mg./Kg. I. V.) Effect on B. P.	Resp. (a)	Miscellaneous (Doses Mg./Kg. I. V. Unless Otherwise Noted)
Su-362	H	100	mS	100	0	100	0	100	0	100	0	50	mS	1-10 S	S	1-10: little effect on B. P. and no effect on uterus of cat *in vivo*.
												100	0	Delayed death		
Su-390	Allylamino	100	0	100	0	100	0	50	0	10	S	400	(I)?	7 0	..	
										50	IS	....	...	.. ...	..	
Su-355	Benzylamino	10	S	100	I	100	P	100	0	10	S	20	0	1 0	..	1-10: no effect on cat uterus *in vivo*, little effect on B. P., inhibition of respiration.
												50	(I)	5 SI	Var.	
														C. N. S.: S?		
Su-446	Phenylamino	100	0	100	I	100	P	100	(S)	10	S	100	(I)	5-10 mI	I	2-10: moderate stimulation of cat uterus *in vivo* with little B. P. change.
												200	II	Rate Decr.		
Su-665	Phenoxymethyl	100	(I)	100	P	100	I	10	I	10	S	200	mI	10 0	0	300: I. P. rats gave convulsions, paralysis, salivation.
								G. P.	..	G. P.	..	Rate Decr.		Rate Incr.		
Su-666	*p*-Methylphenoxymethyl	10	I	100	I	100	I	10	I	10	S	100	(I)?	10 0	0	350: I. P. rats gave ataxia and paralysis.
								G. P.	..	G. P.	..	....	...	.. ...	..	
Su-668	*o*-Isopropylphenoxymethyl	100	(I)	10	I	100	I	1	I	10	S	50	(I)	10 (S)	0	
								G. P.	..	G. P.	..	....	...	Rate Decr.; p. pr.+		
Su-684	*p*-Diphenyloxymethyl	100	(S)	100	I	100	I	10	I	10	S	100	(I)	2.5 mIpp	mI	
								G. P.	..	G. P.	..	200	I	.. ...	..	
												Rate Decr.; Irreg.				
Su-738	*p*-Chlorophenoxymethyl	100	I	100	I	100	I	10	I	100	S	100	(I)	10 (I)	(S)	
								G. P.	..	G. P.	..	Rate Decr.		Rate Decr.; Epi.+?		
Su-672	*m*-Chlorophenoxy	1	I	100	I	10	P	10	I	100	0	50	mI	10 0	0	
								G. P.	..	G. P.	..	....	...	.. ...	..	
Su-667	2,5-Dimethylphenoxymethyl	100	0	10	I	100	I	100	I	10	S	100	(I)	10 0	0	
								G. P.	..	G. P.	..	Rate Decr.		Epi.+?		
Su-687	Thymoxymethyl	10	I	100	I	10	I	100	(I)	100	0	100	(I)?	3 0	0	
		100	0	...	..	...	..	G. P.	..	G. P.	..	....	...	10 (S)	0	
														Epi.+; p. pr.+		
Su-669	N-Ethyl-N-Phenylaminomethyl	100	0	100	I	100	P	10	I	1	S	100	mI	.. ...	..	
								G. P.	..	G. P.	..	....	...	.. ...	..	
Su-664	N-Benzyl-N-Phenylaminomethyl	...	...	...	..	...	..	....	..	...	..	100	0	5 mS	mI	
														10 mSpp	mI	
Su-663	*p*-Methylphenylthiomethyl	100	I	100	P	100	I	10	I	100	S	100	0	5 (I)	0	
								G. P.	..	G. P.	..	....	...	10 (S)	0	
Su-391	Bis-(4-morpholinoethyl-2-thiazolyl)amine	10	I	100	P	100	P	100	0	80	0	200-400	(I)	8 (I)	..	

Explanation of Tables

(*a*) The changes under respiration designate over-all effects on the respiratory volume. When the change was marked, alterations in the rate and depth were usually seen, but no attempt was made to ascribe the change primarily to an alteration in just one of those variables.

(1) These columns summarize the action of the drug on the spontaneous activity of the indicated tissue.

(2) This column summarizes the inhibitory effect, if any, exerted toward the spasmogenic action of 0.6 μg./cc. of histamine dihydrochloride. The drug was added to the bath one minute prior to the addition of the test dose of histamine.

(3) The results in this column are analogous to those described under (2), except the dose of acetylcholine hydrobromide was 0.2 μg./cc.

(4) In all experiments with the isolated heart, the rate was noted and the coronary flow continually measured. The changes noted refer to changes in the amplitude of the kymographically recorded myocardial contractions. No alterations in the rate and coronary flow occurred unless they have been specifically noted. The dose represents the total dose in μg. injected directly into the cannula.

(5) In these experiments the ileum and uterus of the cat were employed unless the designation G. P. is present. In the latter case the duodenum was used in place of the ileum.

Summary of Table Abbreviations

P	= <95% inhibition of response.
I	= >95% inhibition of response. The various forms of "I" have analogous definitions, but connote inhibition or hypotension in respect to blood pressure.
(S)	= Slight stimulation (in respect to blood pressure, a pressor response of less than 10 mm.).
mS	= Moderate stimulation (in respect to blood pressure, a pressor response of 10 to 20 mm.).
S	= Stimulation (in respect to blood pressure, a pressor response of 20 to 40 mm.).
!S	= Marked stimulation (in respect to blood pressure, a pressor response greater than 40 mm.).
p	= Persistent.
pp	= Partially persistent.
0	= No effect.
?	= An effect of questionable significance.
"Epi.−" or "Epi.+"	indicates that the control dose of epinephrine given after the drug elicited, respectively, a lessened or greater than normal response.
B. P.	= Blood pressure
T. V.	= Thiry-Vella.
p. pr.	= Pulse pressure
G. P.	= Guinea pig.
Resp.	= Respiration.
Decr.	= Decreased.
Incr.	= Increased.
Var.	= Variable.
C. N. S.	= Central Nervous System.
I. P.	= Intraperitoneal.
I. V.	= Intravenous.
Irreg.	= Cardiac irregularities.
−	= Diminished.

exert a toxic effect that a wide variety of substituents did not entirely suppress. The moderate antihistaminic activity of some members of the series led to generalizations relating structure with this activity. These confirmed the generalizations concerning antihistaminic action that have already been acknowledged as valid for other series of compounds.

REFERENCES

(1) Djerassi, C., Mizzoni, R., and Scholz, C. R., *J. Org. Chem.*, **15**, 700(1950).
(2) Craver, B. N., Cameron, A., and Barrett, W., *J. Pharmacol. Exptl. Therap.*, **99**, 353(1950).
(3) von Hofmann, A. W., *Ber.*, **12**, 1126, 2359(1879).
(4) Olin, J. F., and Johnson, T. B., *J. Am. Chem. Soc.*, **53**, 1475(1931); (see also earlier papers in bibliography).
(5) Burger, A., and Ullyot, G. E., *J. Org. Chem.*, **12**, 342 (1947).
(6) Di Maggio, G., *Boll. soc. ital. biol. sper.*, **22**, 1081(1946).
(7) Jones, E. R. H., Robinson, F. A., and Strachan, M. N., *J. Chem. Soc.*, **1946**, p. 87.
(8) Lee, H. M., and Jones, R. G., *J. Pharmacol. Exptl. Therap.*, **95**, 71(1949).
(9) Seifter, J., Ehrich, W. E., and Hudyma, G. M., *ibid.*, **92**, 303(1948).
(10) Jeantet, A., *Arch. maladies profess.*, **7**, 9(1946).
(11) Gregoire, C., *Compt. rend. soc. biol.*, **140**, 1218(1946)
(12) Perrault, M., and Bovet, D., *Lancet*, **I**, 731(1946).
(13) Gibbs, E. M., and Robinson, F. A., *J. Chem. Soc.*, **1945**, p. 925.
(14) Vaughan, J. R., Anderson, G. W., Clapp, R. C., Clark J. H., English, J. P., Howard, K. L., Marson, R. W., Sutherland, L. H., and Denton, J. J., *J. Org. Chem.*, **14**, 228(1949).

Investigations of Di(acetylhydroxyphenyl)isatin. I. Quantitative Excretion in Rats*

By R. G. SANDERS, H. M. BAUM, and G. J. STRAUB

Graded doses of di(acetylhydroxyphenyl)-isatin are completely recovered in the feces of the rat within seventy-two hours. No intestinal damage is observable using tests for occult fecal blood as a criterion. Intraperitoneal injections of the subject material in mice are not absorbed, remaining in the peritoneal cavity as inert foreign bodies. Animals receiving excessive doses exhibit diarrhea and some hyperirritability.

THE DIPHENYL ISATINS are synthetic materials which were chemically investigated by Baeyer and Lazarus in 1885 (1). Some time later, Guggenheim (2) synthetically prepared di-(acetylhydroxyphenyl)isatin (Isacen). He first tried this material on himself and found that it acted as an aperient and laxative without untoward effects. It was subsequently studied clinically by Katzenelbogen and Guder (3), by Einhorn and Rafsky (4), and others. European workers, studying the effect of smaller doses of Isacen on dogs, reported only laxative properties and no toxic reaction.

Hydroxy derivatives of anthraquinone occur in plants both as the glycoside and in the free state, and constitute the laxative principle of such widely used natural materials as cascara, rhubarb, senna, and aloes. Isacen is chemically similar to naturally occurring hydroxyanthraquinone glycosides in that it contains the group $(HO—C_6H_4)_2{=}C{=}$, which has been ascribed as responsible for laxative activity by Kaufman (5) and Easson, *et al.* (6).

It thus became of interest to study the effect of massive doses of di(acetylhydroxyphenyl)isatin as one phase of a basic investigation of its laxative and cathartic properties.

EXPERIMENTAL

Twenty young female rats weighing between 70 and 75 Gm. were placed in individual cages, divided into groups of four and fasted for twenty-four hours. Each animal of the group was respectively intubated with 1.0, 2.0, 4.0, 8.0, and 16.0 mg. of Isacen dispersed in gum tragacanth. The animals were then given food *ad libitum* and observed for gross symptoms. Severe diarrhea occurred within four hours in the groups receiving 4.0, 8.0, and 16.0 mg.; the groups receiving 1.0 and 2.0 mg. exhibited diarrhea within the first twenty-four hours. All stools of diarrheal nature were tested by classical benzidine methods for occult blood. None was found present. Animals receiving 8.0- and 16.0-mg. doses exhibited hyperactive reflexes to noise and motion, which seemed to coincide with diarrhea. All animals appeared normal after seventy-two hours, and were subsequently raised to maturity.

Metabolism Studies.—Six young female rats weighing approximately 115 Gm. were placed in metabolism cages and fasted for twenty-four hours. Two animals were intubated with a blank dispersion of tragacanth and water as controls; two others with 4.0 mg. each, and the remaining with 8.0 mg. of Isacen. The feces were collected quantitatively for seventy-two hours, dried, and extracted with chloroform in a Soxhlet. Extreme difficulty was encountered in the extraction, ninety-six hours being required. The chloroform extracts were then analyzed for Isacen by evaporating to dryness, diluting with alcohol, and treating with sodium hydroxide and potassium ferricyanide solution. The red color produced behaves according to Beer's law and was read in a Coleman Spectrophotometer against a known standard solution. The results are summarized in Table I.

TABLE I

Animal No.	Isatin Dose, Mg.	Isatin Recovery, %	Occult Blood	General Observations
638	4.0	97–98	Neg.	Diarrhea
643	4.0	97–98	Neg.	Diarrhea
669	8.0	96–98	Neg.	Diarrhea
641	8.0	96–98	Neg.	Diarrhea
625	16.0	>90[b]	Neg.	Diarrhea
639[a]	0	0	Neg.	None
627	16.0	>90[b]	Neg.	Diarrhea
673[a]	0	0	Neg.	None

[a] Control—blank.
[b] Part of material lost during final extraction.

Intraperitoneal Injections in Mice.—Four adult mice were injected intraperitoneally with 0.5 cc. of a tragacanth dispersion containing 4.0 mg. of Isacen. The animals were sacrificed and autopsied after twenty-four hours. The material was dispersed in the peritoneal cavity of all the animals, and presented a white amorphous appearance. There was no evidence of irritation to the peritoneum, even in areas where the Isacen was in direct contact with the membrane. The material behaved as an inert foreign body.

DISCUSSION

The effective clinical dose of di(acetylhydroxyphenyl)isatin has been reported as 5.0 mg. for a 70-Kg. man. In our experiments the dose in the 16.0-mg. intubation was equivalent to 132.0 mg./Kg.

* Received January 29, 1951, from the Biological Research Division, Harrower Laboratories, St. Louis, Mo.

In all levels administered a definite laxative effect was observed. The twenty-four hour fasting of the animals was designed to produce a maximum laxative effect for a given dose. It is considered significant that the effect produced on the intestinal mucosa was not severe enough to demonstrate occult fecal blood in any instance. Of further interest is the fact that di(acetylhydroxyphenyl)isatin is quantitatively excreted within seventy-two hours in the feces, which is in marked contrast to the action of phenolphthalein, of which approximately 15% of ingested material is reported to be absorbed through the wall of the alimentary canal and excreted through the kidney (7, 8).

SUMMARY AND CONCLUSIONS

1. The complete recovery of doses up to 16.0 mg. of di(acetylhydroxyphenyl)isatin has been demonstrated in the feces of rats.

2. No adverse effects of the material, except a transitory hyperirritability of a minor nature accompanying the diarrhea, were noted.

REFERENCES

(1) Baeyer, A., and Lazarus, M. S., *Ber.*, **18**, 2637(1885).
(2) Guggenheim, M., *Schweiz. med. Wochschr.*, **55**, 16 (1925).
(3) Katzenelbogen, S., and Guder, R., *ibid.*, **55**, 18(1925).
(4) Einhorn, M., and Rafsky, H. A., *J. Am. Med. Assoc.*, **86**, 1754(1926).
(5) Kaufman, Z., *Angew. Chem.*, **40**, 831(1937).
(6) Easson, A. P. T., Harrison, J., McSwinney, B. A., and Pyman, F. L., *Quart. J. Pharm. Pharmacol.*, **7**, 509 (1934).
(7) Sollman, T., "Manual of Pharmacology," 7th ed., W. B. Saunders Co., Philadelphia, 1948, p. 177.
(8) Goodman, L., and Gilman, A., "Pharmacological Basis of Therapeutics," Macmillan Co., New York, 1941.

A Procedure for the Isotopic Synthesis of *Alpha*-Amino Acids with Reference to Isoleucine*

By GEORGE B. CERESIA†, G. L. JENKINS, and ED. F. DEGERING

A procedure for the synthesis of isotopic *dl*-isoleucine is described wherein a labeled carbon atom can be incorporated as a methyl group in the β-position of the chain. A study of four aliphatic alcohols (*sec*-butyl, *n*-amyl, iso-amyl, and *n*-octyl alcohols), to ascertain the influence of alkyl chains on each synthetic step, gave no significant results. The following new compounds were prepared: *sec*-butoxymethyl ethyl ketone, *n*-amyloxymethyl ethyl ketone, *sec*-butoxy-2-methyl-2-butanol, iso-amyloxy-2-methyl-2-butanol, *n*-amyloxy-2-methyl-2-butanol, and *n*-octyloxy-2-methyl-2-butanol.

With the greatly increased availability of isotopic carbon, particularly C^{14}, since World War II in the form of barium carbonate, cyanide salt, and methyl iodide, its use as a means of medical detection and research, particularly in cancer, has also multiplied. Consequently, much thought and effort has been and is still being expended on synthesis of compounds with a labeled carbon atom.

The radioactive isotopic α-amino acids are very important in studies of the cause, growth, and malignant effects of cancer. From their beta rays, they can be detected, followed, and quantitatively determined as metabolites by the Geiger-Mueller counter (1) without sacrifice or discomfort to the animal. The possibility also exists that, like the curative power of radioactive iodine on cancer of the thyroid gland, radioactive carbon compounds may alleviate and perhaps cure certain types of cancer.

Therefore, the problem was to select and devise procedures which would not only insure a maximum yield under semimicro conditions, but would also allow the unreacted isotopic element to be recovered quantitatively. Likewise, since the isotopic carbon may be incorporated anywhere but in the metabolically labile carboxyl group, the ideal procedure is to add it in the last synthetic step; for isoleucine the β-methyl would be best. Unfortunately, no such procedure was found or could be devised.

* Received May 18, 1951, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Ind.
† Indiana Elks Association Research Fellow, 1949–1950.

METHOD

Although several methods of synthesis were investigated, the following method gave the best yields:

$$ROCH_2COC_2H_5 + \overset{*}{C}H_3MgI \rightarrow ROCH_2C(\overset{*}{C}H_3)OHC_2H_5$$

$$ROCH_2C(\overset{*}{C}H_3)OHC_2H_5 + H^+ \rightarrow C_2H_5C(\overset{*}{C}H_3)HCHO + ROH$$

$$C_2H_5C(\overset{*}{C}H_3)HCHO + HCN \rightarrow C_2H_5C(\overset{*}{C}H_3)HC(OH)HCN$$

$$C_2H_5C(\overset{*}{C}H_3)HC(OH)HCN + (NH_4)_2CO_3 \rightarrow C_2H_5C(\overset{*}{C}H_3)HC\begin{matrix}CO-NH\\ \diagup\quad\quad|\\ \diagdown\quad\quad|\\ NH-CO\end{matrix}$$

$$\text{and then} + Ba(OH)_2 + H_2O \rightarrow C_2H_5C(\overset{*}{C}H_3)HC(NH_2)HCO_2^-\tfrac{1}{2}Ba^+$$

In this instance the carrier for the isotopic carbon is methyl iodide, which is readily available and can be prepared in 80–90% yield from labeled barium carbonate (2, 3).

Accordingly, for the purpose of studying the limitations of the reaction and the extent of the yields, the alkoxymethyl ethyl ketones were prepared in the following manner (4–6):

$$ROH + CH_2O + HCl \rightarrow ROCH_2Cl$$
$$ROCH_2Cl + CuCN \rightarrow ROCH_2CN$$
$$ROCH_2CN + C_2H_5MgBr \rightarrow ROCH_2COC_2H_5$$

In chloromethylation of the alcohol, trioxan was employed as the source of formaldehyde and gave a better yield than paraformaldehyde, although Farren, *et al.* (7), and Henze, *et al.* (6), reported yields as high as 70% with paraformaldehyde. If sufficient time is allowed so that the acetals formed are split and converted to alkoxy chloromethyl ethers, the yields are 85–91%.

Four aliphatic alcohols—*sec*-butyl, *n*-amyl, isoamyl, and *n*-octyl alcohol—were selected to show the influence of alkyl chains on the yield of each synthetic step. They were selected, also, to show the ease of proton-initiated rearrangement of the corresponding tertiary alcohols. The differences reported in Tables I, II, III, and IV were not sufficiently significant to draw any valid conclusions.

The alkoxyacetonitrile was made after the manner of Gauthier (8), which is the replacement of the chlorine atom in the α-position of the chloro ethers by the nitrile, through the use of CuCN or HgCN in yields of 59–81%.

Blaise (9) discovered that nitriles are converted through the action of alkylmagnesium halides into ketones, and this was followed by the conversion of alkoxy nitriles into ketones by use of the Grignard reagent (10). Accordingly, the alkoxymethyl ethyl ketones were made by treating an equivalent of alkoxyacetonitrile with a 10% excess of ethylmagnesium bromide, cooling, hydrolyzing, separating, washing the ethereal solution with 5% sodium hydroxide and distilled water, and drying. Barnes and Budde (4) state that if the solution of Grignard and alkoxyacetonitrile are maintained at −50 to −40°, there is approximately a 15% increase in yield of ketone. This was tried and the yield of the iso-amyloxymethyl ethyl ketone rose from 46 to 53%. Even this figure, however, does not measure up to the claims of Rigler and Henze (5), who obtain 82% by the same method. Their yields of alkoxy ketones are in the range of 71–84%, whereas yields shown in Table III agree more closely with those obtained by Price (11) and Barnes and Budde.

The alkoxy tertiary alcohols are made by treating the corresponding ketone with a 10% excess of Grignard reagent—in this case methylmagnesium iodide. However, since the methyl Grignard contains the labeled carbon atom, the reverse was done and the yield improved by cooling and adding an excess of ketone. The procedure for synthesis of the tertiary alcohols is the same as that for synthesizing the alkoxy ketones, with the exception that it is very important to wash the ethereal solution with 5% aqueous sodium hydroxide and distilled water. If this is not done, free iodine is liberated upon stand-

TABLE I.—CHLOROMETHYL ETHERS, $ROCH_2Cl$

R	Conversion, %	B. P., °C.	n^{25}_D
sec-Butyl	60	119	....
n-Amyl	80	61/30 mm.	1.4252
Iso-amyl	76	154/75 mm.	1.425
n-Octyl	66	77–79/6 mm.	1.4363

TABLE II.—ALKOXYACETONITRILES, $ROCH_2CN$

RO—	Yield, %	B. P., °C.	n^{25}_D
sec-Butoxy	58.5	72–73/28 mm.	1.4066
n-Amyloxy	90.0	77/15 mm.	1.4149
Iso-amyloxy	81.0	183–184/752 mm.	1.4140
n-Octyloxy	80.9	105–106/11 mm.	1.4283

TABLE III.—ALKOXYMETHYL ETHYL KETONES, $ROCH_2COC_2H_5$

RO—	Yield, %	B. P., °C.	n^{25}_D
sec-Butyloxy	46.9	52–53/7 mm.	1.4152
n-Amyloxy	51.7	68–69/7 mm.	1.4218
Iso-amyloxy	53.0	196–197/752 mm.	1.4192
n-Octyloxy	49.3	94–95/5.6 mm.	1.4290

TABLE IV.—ALKOXY-2-METHYL-2-BUTANOL, $ROCH_2C(CH_3)OHC_2H_5$

RO—	Yield, %	B. P., °C.	n^{25}_D
sec-Butoxy	58.4	53.5/10 mm.	1.4200
n-Amyloxy	68.7	72/8 mm.	1.4260
Iso-amyloxy	62.5	60/5.5 mm.	1.4195
n-Octyloxy	42.1	104/5 mm.	1.4346

ing and causes side reactions as well as partial rearrangement.

The rearrangement of the alkoxy tertiary alcohol to the corresponding aldehyde closely parallels the pinacol-pinacolone shift involving a carbonium ion (12). The problem is, therefore, to obtain the best conditions and select the best type of acid as a proton donor. Barnes and Budde used 10% hydrochloric acid and anhydrous oxalic acid. They obtained the desired aldehydes in yields of 60–75%. Elphimoff used anhydrous formic acid to rearrange α-alkoxy tertiary alcohols of the type $RCH(OR)C(OH)Me_2$ to the corresponding aldehydes (13). Price used oxalic acid dihydrate and dilute mineral acids and obtained yields as high as 75%. As a result four acids (10% acetic, oxalic dihydrate, anhydrous oxalic, and a trace of hydriodic) were tried. Anhydrous oxalic acid proved the best and gave a yield of 69.3%.

Gaudry (14) was able to improve the Strecker method (15) of synthesizing amino acids by using sodium cyanide and ammonium carbonate on the sodium bisulfite addition product of the aldehyde. Thus the yield was raised from 56 to 84%.

The hydantoin should be continuously extracted with ethyl ether for one to two days before treatment with ammonium carbonate. Price found that less time was consumed if the cyanohydrin was formed directly from the aldehyde and hydrogen cyanide. The optimum yield (83%) of 5-(2-methylbutyl)-hydantoin was realized when 0.25 mole of 2-methylbutanal, 0.26 mole of hydrogen cyanide, and 0.6 mole of ammonium carbonate were used.

The aldehyde was determined gravimetrically by reaction of an aliquot of impure aldehyde (less than 0.01 mole) with 3 Gm. (0.011 mole) of 2,4-dinitrophenylhydrazine in 150 ml. of 95% ethanol.

Investigation of the literature reveals that iso-amyloxy-2-methyl-2-butanol, *n*-octyloxy-2-methyl-2-butanol, *n*-amyloxymethyl ethyl ketone, *sec*-butoxymethyl ethyl ketone, and their corresponding tertiary alcohols are new compounds.

EXPERIMENTAL

Chloromethyl Ethers.—*sec*-Butyl, *n*-amyl, and *n*-octyl chloromethyl ethers were prepared according to the procedure described for iso-amyl chloromethyl ether.

Iso-amyl Chloromethyl Ether.—In a 1-L. three-necked flask equipped with a Hershberg stirrer, a thermometer, and a gas inlet tube, dry hydrogen chloride is passed into a mixture of 440 Gm. (5 moles) of iso-amyl alcohol and 150 Gm. (5.3 moles) of trioxan, for eighteen hours with agitation and at 0 to 15°. Separate the top oily layer and dry over anhydrous calcium chloride for two days to remove the water and unreacted alcohol. Distill *in vacuo* in a large bore distillation apparatus to prevent the paraformaldehyde (which precipitates out) from clogging the apparatus. Save the higher boiling fraction, which contains acetal, for further treatment with hydrogen chloride. Rectify and collect the fraction boiling at 154°. The yield is 518 Gm. (76% conversion).

Alkoxyacetonitriles.—*sec*-Butoxy, *n*-amyloxy, and *n*-octyloxyacetonitriles are synthesized in the same manner as iso-amyloxyacetonitrile.

Iso-amyloxyacetonitrile.—Add 180 Gm. (2.0 moles) of powdered cuprous cyanide slowly to 273 Gm. (2.0 moles) of iso-amyl chloromethyl ether in a 1-L. three-necked flask equipped with Hershberg stirrer, thermometer, and powder funnel, with agitation. Do not let the temperature exceed 90°. When the reaction subsides, heat the flask to 110–120° for two hours, cool to room temperature, and decant the clear liquid. Distill under vacuum and rectify the distillate. Collect the fraction boiling at 183–184° (752 mm.). The yield is 208 Gm. (81.0%).

Alkoxymethyl Ethyl Ketones.—*n*-Octyloxymethyl ethyl ketone is prepared in the same manner as that described for iso-amyloxymethyl ethyl ketone.

Iso-amyloxymethyl Ethyl Ketone.—Prepare 1.7 moles of ethylmagnesium bromide from 40.8 Gm. of Mg and 185.5 Gm. of ethyl bromide in the usual manner, in a 1-L. three-necked flask equipped with a Hershberg stirrer, dropping funnel, and reflux condenser. Agitate the solution vigorously and immerse the flask in a gallon Dewar flask with dry ice trichloroethylene mixture at −50°, just before adding 190.5 Gm. (1.5 moles) of iso-amyloxyacetonitrile. Add the acetonitrile rapidly and then remove the Dewar flask. Agitate for one hour and allow the mixture to stand overnight. Then decant the liquid from the excess of magnesium turnings into a 1-L. beaker immersed in ice water and carefully hydrolyze with cold distilled water and 50% sulfuric acid. Wash the ethereal layer with 5% sodium hydroxide and then distilled water until neutral to hydrion paper. Dry the product first with anhydrous sodium sulfate and finally with Drierite for twenty-four hours. Flash the ether off with a water aspirator and rectify. Collect the fraction boiling at 196–197° (752 mm.). The yield is 138.0 Gm. (54%).

***sec*-Butoxymethyl Ethyl Ketone.**—This compound is prepared in a similar manner from 1.2 moles of ethylmagnesium bromide (28.8 Gm. of Mg and 130.8 Gm. of ethyl bromide in 300 ml. of anhydrous ether) and 113 Gm. (1.0 mole) of *sec*-butoxyacetonitrile in 300 ml. of anhydrous ether. The fraction boiling at 52–53° (7 mm.) and with an index of refraction ($n^{20.5}_D$) 1.4152 is collected. The yield is 67.5 Gm. (46.9%).

Anal.—Calcd. for $C_9H_{18}O_2$: C, 66.2; H, 11.2. Found: C, 66.4; H, 11.3.

***n*-Amyloxymethyl Ethyl Ketone.**—This ketone is prepared in a like manner from 1.3 moles of ethylmagnesium bromide (31.2 Gm. of Mg and 115.7 Gm. of ethyl bromide in 300 ml. of anhydrous ether) and 149.7 Gm. (1.1 moles) of *n*-amyloxyacetonitrile dissolved in 300 ml. of anhydrous ether. The fraction boiling at 68–69° (7 mm.) and with an index of refraction ($n^{20.5}_D$) 1.4218 is collected. The yield is 88.8 Gm. (51.7%).

Anal.—Calcd. for $C_9H_{18}O_2$: C, 68.26; H, 11.38. Found: C, 68.1; H, 11.22.

Iso-amyloxy-2-Methyl-2-Butanol.—Prepare 1.0 mole of methylmagnesium iodide (26.4 Gm. of Mg and 142 Gm. of freshly distilled, dry methyl iodide dissolved in 300 ml. of anhydrous ether) in the usual manner, in a 1-L. three-necked flask equipped with a Hershberg stirrer, dropping funnel, and reflux condenser. Then add 173.8 Gm. (1.1 moles) of iso-amyloxymethyl ethyl ketone dissolved in 300 ml. of anhydrous ether to keep a constant reflux under

agitation. When complete, reflux one hour and allow the reaction to stand overnight. Then follow the same procedure as described for iso-amyloxymethyl ethyl ketone. The fraction boiling at 60° (5.5 mm.) and with an index of refraction (n^{20}_D 1.4195 is collected. The yield is 119.5 Gm. (62.5%).

Anal.—Calcd. for $C_{10}H_{22}O_2$: C, 68.9; H, 12.64. Found: C, 69.2; H, 12.7.

***sec*-Butoxy-2-Methyl-2-Butanol.**—This compound is prepared in a similar manner from 0.46 mole of methylmagnesium iodide and 65 Gm. (0.45 mole) of *sec*-butoxymethyl ethyl ketone. The fraction boiling at 53.5° (10 mm.) and with an index of refraction (n^{20}_D 1.4200 is collected. The yield is 41.9 Gm. (58.4%).

Anal.—Calcd. for $C_9H_{20}O_2$: C, 67.4; H, 12.5. Found: C, 67.68; H, 12.38.

***n*-Amyloxy-2-Methyl-2-Butanol.**—This alcohol is prepared in a like manner from 0.5 mole of methylmagnesium iodide and 86.9 Gm. (0.55 mole) of *n*-amyloxymethyl ethyl ketone. The fraction boiling at 71–72° (8 mm.) and with an index of refraction (n^{20}_D 1.4260 is collected. The yield is 65.6 Gm. (68.7%).

Anal.—Calcd. for $C_{10}H_{22}O_2$: C, 68.9; H, 12.64. Found: C, 68.8; H, 12.85.

***n*-Octyloxy-2-Methyl-2-Butanol.**—This compound is prepared in a manner similar to that described for *n*-amyloxy-2-methyl-2-butanol. The fraction boiling at 104° (5 mm.) and with an index of refraction (n^{20}_D 1.4346 is collected. The yield is 49.8 Gm. (42.1%).

Anal.—Calcd. for $C_{13}H_{28}O_2$: C, 72.24; H, 12.98. Found: C, 72.0; H, 12.8.

2-Methylbutanal.—A mixture of 0.05 mole of *n*-amyloxy-2-methyl-2-butanol (8.7 Gm.) and 0.05 mole of anhydrous oxalic acid (5.0 Gm.) is refluxed four hours at 105–110° and the contents of the flask and rinsings of the condenser are washed into a 250-ml. volumetric flask. Dilute to mark with 50% ethanol and react a 50-ml. aliquot with 2,4-dinitrophenylhydrazine (3 Gm. in 150 ml. of 95% ethanol; 0.011 mole) by heating the mixture to a boil, adding 3 ml. of concentrated hydrochloric acid and again bringing to a boil. The mixture is boiled for five minutes and then placed in the refrigerator overnight. A standard consisting of 3 Gm. of freshly distilled, dry iso-butyraldehyde in a similar amount of 2,4-dinitrophenylhydrazine solution is run in conjunction with the unknown. The yield of unknown hydrazone multiplied by the correction factor 1.196 gives 69.3% of aldehyde.

The same procedure is followed for rearrangement by 10% acetic acid, oxalic acid dihydrate, and 50% hydriodic acid, with the exceptions that an equivalent amount of acetic is used to an equivalent amount of the alkoxytertiary alcohol and only five drops, or approximately 0.005 mole, of hydriodic acid is used to 0.046 mole of the alkoxytertiary alcohol. The yields are 18.5%, 41%, and a trace, respectively.

5-(2-Methylbutyl)hydantoin.—Add 8.6 Gm. (0.1 mole) of 2-methylbutanal, dropwise, to a cooled, stirred solution of 10.5 Gm. (0.1 mole) of sodium bisulfite in 50 ml. of water. Then add slowly with agitation 6.6 Gm. (0.1 mole) of potassium cyanide, dissolved in 50 ml. of water, to the cooled sodium bisulfite paste and stir one hour at room temperature. Extract with ethyl ether for thirty-six hours with a continuous extractor and evaporate the ether off. Add the resulting cyanohydrin to 23 Gm. (0.24 mole) of ammonium carbonate in 50 ml. of water and maintain the mixture at 50° for five hours. Then heat to 90–100° for one hour to remove the excess ammonium carbonate, filter while hot, and concentrate the solution to a small volume with a water aspirator. On cooling, needles crystallize out and the solid is purified by recrystallizations from hot water. The yield is 129.5 Gm. (83%).

***dl*-Isoleucine.**—Dissolve 15.6 Gm. (0.1 mole) of recrystallized 5-(2-methylbutyl)hydantoin and 47.5 Gm. (0.15 mole) of barium hydroxide octahydrate in 100 ml. of boiling water and transfer the solution to a Carius tube. Seal and heat the tube to 150–160° for one to two hours. Then cool to room temperature, filter the contents, and wash the precipitate with hot water. Remove the barium with ammonium carbonate and boil the solution to remove the excess ammonium carbonate. Filter hot, evaporate the filtrate over a steam cone under vacuum, and dissolve the residue in a minimum of hot water with an equal volume of ethanol added. Place in a refrigerator overnight. The yield is 9.83 to 10.14 Gm. (75–85%).

REFERENCES

(1) Kamen, M. D., "Radioactive Tracers in Biology," Academic Press, Inc., New York, 1948.
(2) Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., "Isotopic Carbon," John Wiley and Sons, Inc., New York, 1949.
(3) Tolbert, B. M., *J. Am. Chem. Soc.*, **69**, 1329(1947).
(4) Barnes, R. A., and Budde, W. M., *ibid.*, **68**, 2339(1946).
(5) Rigler, N. E., and Henze, H. R., *ibid.*, **56**, 474(1936).
(6) Henze, H. R., Duff, V. B., Matthews, W. H., Jr., and Forman, E. D., *ibid.*, **64**, 1222(1942).
(7) Farren, J. W., Fife, H. R., Clark, F. E., and Garlan C. E., *ibid.*, **47**, 2419(1925).
(8) Gauthier, M. D., *Compt. rend.*, **143**, 831(1906); *Ann. chim. phys.*, **16**, 289(1909).
(9) Blaise, E. E., *ibid.*, **132**, 28(1901).
(10) Behal, A., and Sommelet, M., *Bull. soc. chim.*, **31**, 303 (1904).
(11) Price, H. S., "A Procedure for the Synthesis of *dl*-Valine Containing a Labeled Carbon Atom," Ph.D. thesis, Purdue University, June, 1950.
(12) Wheland, G. W., "Advanced Organic 1949, Chemistry," 2nd ed., John Wiley and Sons, Inc., New York, 1949, pp. 451–475.
(13) Elphimoff, I., *Compt. rend.*, **224**, 399(1947).
(14) Gaudry, R., *Can. J. Research*, **24**, 301(1946).
(15) "Organic Syntheses," Coll. Vol. I and II, John Wiley and Sons, Inc., New York, 1941, 1947.

A Method for Biologic Standardization of *Veratrum viride* Based on the Survival Time of *Daphnia magna**

By R. O. CRAW† and ALAN E. TRELOAR‡, §

A method for biologic standardization of *Veratrum viride* has been devised by measuring toxicity of the water-soluble principles on *Daphnia magna*. The method is based on survival time of the test animal when immersed in 10 per cent w/v aqueous extracts. Applicability of the assay method to routine control procedures is described. Statistical analysis of data indicates that the method is dependable and that results obtained by one technician can be duplicated by another.

THE PRESENT report is of an interim character, offered because of the desirability for publication of the present basis of standardization of commercial products containing whole powdered *Veratrum viride*. Extensive exploratory studies are not detailed herein.

Methods for standardization of *Veratrum viride* preparations based on chemical analysis have not been practical (1–4). Until the constitution of the drug with respect to its complex of active principles is more fully defined, other methods of standardization must be used.

A number of workers (5–9) have proposed biologic methods for evaluating the toxicity of *Veratrum viride* extracts using various laboratory animals. None of these assay methods, however, has been officially adopted (10).

The value of biologic assay has been greatly increased in recent years through the contributions of statisticians in indicating methods of control achievable through better design of experiments and appropriate analysis of results. Certain procedures have received considerable attention, to a point where one might say they have themselves become standards. Other procedures will be developed to meet different needs as they are recognized. This paper is a contribution in the latter direction, growing out of attempts to devise a simple procedure for the bioassay of *Veratrum viride*, using large numbers of an easily cultured experimental animal.

EXPERIMENTAL

Daphnia magna and Bioassay.—*Daphnia magna*, a small fresh-water crustacean, was selected as a test animal for this investigation.

Extensive work by Viehoever and co-workers (11–17), has shown that this animal is a valuable "living reagent" for measurement of drug action. Viehoever was also able to demonstrate that the principal systems of *Daphnia magna* resemble, in many respects, the principal systems of the vertebrate. Furthermore, Baylor (18) concluded that the *Daphnia magna* heart, like that of the vertebrate, is inhibited by acetylcholine and by potassium. He was also able to demonstrate that there was a direct relation between heart rate and drug concentration. Obreshkove (19) demonstrated that acetylcholine increased peristalsis of the intestine in *Daphnia magna*, which is another indication of the similarity of the cholinergic systems of *Daphnia magna* and the vertebrates.

From the point of view of biological characteristics, there is much to commend *Daphnia magna* as an experimental animal for assay purposes. To this may be added two other factors of considerable importance in such work:

(*a*) Animals may be propagated readily and economically *in large numbers* under highly standardized conditions.

(*b*) Transparency of *Daphnia magna* enables activity of the principal organs and systems to be observed microscopically without resorting to operative techniques.

The research to be reported in this paper was undertaken initially because of the cogency of evidence and supporting argument given above concerning the potentialities of *Daphnia magna* as a test animal. It has subsequently been deemed worthy of persistent pursuit.

Culture and Standardization of Daphnia magna.—The culture medium is prepared by suspending 5 Gm. of prepared cow manure (Bovung) and 5 Gm. of prepared sheep manure (garden variety) in 10 L. of tap water.[1] This is allowed to stand for three days, during which time all free chlorine and chloramines are eliminated in the presence of organic matter (negative *ortho*-tolidine test). The medium is then ready for use.

Daphnia magna normally reproduces asexually. On the ninth day of life, eggs contained in the egg chamber of the adult animal pass into the embryo stage, and on the tenth day, young are released. Thereafter, young animals are released every three days. The colony used in this investigation is maintained as follows: propagation is carried out by placing gravid females from a stock culture, by

* Received September 11, 1950, from the Control Department of Irwin, Neisler & Co., Decatur, Ill.

† Staff Member, Research Laboratories, Irwin, Neisler & Co., Decatur, Ill.

‡ Professor of Biostatistics, University of Minnesota.

§ The authors wish to acknowledge the technical assistance, in experimental work, of Martha D. Napoli, and the assistance of Dr. H. W. Dorn, Director of Research, in compiling material used in this paper.

[1] Tap water in this locality has the following approximate analysis given in parts per million: calcium 40–90, chlorides 6, sulfates 20–70, phosphates less than 1, insolubles 0.5, iron and fluorine negligible.

means of a pipette, one in each of 1-oz. round wide-mouthed bottles three-fourths filled with medium. All bottles are checked daily for appearance of young. Newly released animals are separated from the parent animal by means of a pipette. Immediately thereafter, sets of 100 young are accumulated in 4-L., round, wide-mouthed jars containing 1 L. of fresh medium. Identified by date, these jars are subsequently given 200 cc. of fresh medium daily. Productive females are transferred to fresh medium and the nonproductive animals are discarded.

Test animals are propagated in a constant temperature room which is maintained at 72° F. ± 2°. Culture medium is also prepared and kept in this room.

Standard Extract of Veratrum viride.—In light of the observations of Viehoever, and more recently Noonan (20), that toxic principles of *Veratrum viride* were extractable with water, and since aqueous media are the normal environment of *Daphnia*, it was felt that use of aqueous *Veratrum viride* extracts would be more adaptable than nonaqueous extracts to assay purposes. The aqueous extract has accordingly been adopted in this investigation.

Fresh extracts of the drug are prepared for each test during the day preceding that on which the test is to be run. The procedure is as follows: an aqueous 10% w/v suspension of the whole-powdered drug (random sample) is agitated mechanically for approximately four hours and is then allowed to macerate at 72° F. ± 2° for approximately sixteen hours. After filtration, the extract is ready for use.

Assay Procedure.—One ten-day-old female *Daphnia magna*, freed from excess culture medium by pipetting onto a cloth strainer, is placed in a covered Petri dish containing 10 cc. of extract. This is repeated at three-minute intervals, selecting animals of uniform size, until 10 consecutively numbered replicates in separate dishes are prepared. Each animal is examined subsequently at intervals of precisely thirty minutes from the time of its immersion in extract for test, until observations are terminated. Animals that are motionless to the unaided eye are placed on a slide by means of a pipette and, with a beam of cold light passing through the transparent body, the animal heart is studied using a binocular wide-field microscope having a magnification of 112.5. Animals are considered dead when the heart is found to be in systolic standstill.

All assay work is conducted in the special laboratory kept at 72° F. ± 2°. Each 10-animal unit of assay work completed in a single run constitutes one test. Two tests are completed on each testing day, one in the morning and one in the afternoon. Twenty tests in all, occupying ten testing days, are accepted as completion of the assay for each new drug lot in present routine procedure.

Each test is terminated at the end of that thirty-minute period (after test start) in which the total number of deaths reaches five or more. Median length of life for the 10 test animals may then be computed, and this is accepted as the representative test value. Extensive study of this assay technique in our exploration work has shown that median length of life is almost as reliable a test value as mean length of life, and has the decided advantage of requiring approximately 40% less observation time per test that when means are secured. The average of the 20 test values is accepted as the assay value of each drug lot.

RESULTS

Table I presents a typical set of records arising from a routine assay at the present time. Calculations for the median are given by way of illustration. In test 1, four animals were dead at the end of two and one-half hours, one more being needed to reach the median. In the next half-hour, three animals died. Assuming they died at regular intervals through that half-hour period, we take one-third of the half-hour as the increment to add to two and one-half hours, giving 2.67 hours as the median length of life. The average of the 20 test values for D. L.[1] 175 (Table I) gives an assay value of 1.87.

Dependability of Assay Value.—The closeness with which assay values may be reproduced in repetition of assay is, of course, a crucial matter. In Table I, the 20 test values with an average of 1.87 have a variance of 0.0918 and a "root variance"[2] of 0.303. The standard error for this assay value is, therefore, $0.303 \div \sqrt{20} = 0.070$. This measure of sampling variability of the assay value is 3.7% of the assay value of 1.87. We can be reasonably sure from this assay that the entire drug lot 175 has a true value between 1.72 and 2.02, which limits define the 95% confidence interval in this estimation.

Technician Effects.—Changes in personnel recently commended rerunning an assay with technician B so as to allow comparison of her results with those secured approximately twelve months earlier by technician A. A drug lot designated 155 was chosen for this purpose. The outcome was reassuring, the assay values being 2.52 for technician A and 2.42 for technician B. The standard errors of assay value are 0.054 and 0.095 respectively, or 2.1 and 4.0% of the assay values. The difference in assay values is well within errors of random sampling. It will be noted that the newer technician of less experience had good dependability even though she encountered variation of test values nearly twice that found by technician A in this assay. Any significance which one might attach to this difference in variability (the F ratio lies approximately at the 2% point) disappears in considering the wider experience of technician A. The variability values on D. L. 155 happen to be opposite extremes within the records of each technician, 0.054 being a low variability for A, and 0.095 being a high variability within the shorter experience of B.

A major problem in reduction of variation in this assay procedure had been that of highly significant differentiation of the test values arising from work on different days. The results given in Table I show little evidence of this, and it is apparent that considerable progress in control of it has been made.

---

[1] Drug lot.

[2] The term "root variance" is suggested for the "optimum estimate of the standard deviation," as defined by Fisher, to avoid confusion with Pearson's definition of the standard deviation established much earlier

TABLE I.—ROUTINE ASSAY RESULTS FOR *Veratrum viride* LOT 175

Test No.	Animals Killed, in 1/3 Hr. Ending 1	1.5	2	2.5	3	Calculations for Median	Paired Daily Dif.	Tests Av.
1	.	2	0	2	3	$2.5 + (1/3 \times 0.5) = 2.67$	0.92	2.21
2	.	3	4	.	.	$1.5 + (3/4 \times 0.5) = 1.75$		
3	.	2	5	.	.	$1.5 + (3/5 \times 0.5) = 1.80$	0.05	1.78
4	.	3	4	.	.	$1.5 + (3/4 \times 0.5) = 1.75$		
5	.	2	1	3	.	$2.0 + (2/3 \times 0.5) = 2.33$	0.45	2.10
6	.	2	4	.	.	$1.5 + (3/4 \times 0.5) = 1.88$		
7	.	3	3	.	.	$1.5 + (2/3 \times 0.5) = 1.83$	0.08	1.79
8	.	4	2	.	.	$1.5 + (1/2 \times 0.5) = 1.75$		
9	.	3	4	.	.	$1.5 + (3/4 \times 0.5) = 1.75$	0.08	1.71
10	1	3	3	.	.	$1.5 + (1/3 \times 0.5) = 1.67$		
11	1	3	2	.	.	$1.5 + (1/2 \times 0.5) = 1.75$	−0.13	1.82
12	.	2	4	.	.	$1.5 + (3/4 \times 0.5) = 1.88$		
13	.	5	.	.	.	$1.5 + (0 \times 0.5) = 1.50$	0.17	1.42
14	1	6	.	.	.	$1.0 + (4/6 \times 0.5) = 1.33$		
15	.	.	4	2	.	$2.0 + (1/2 \times 0.5) = 2.25$	0.50	2.00
16	1	2	4	.	.	$1.5 + (3/4 \times 0.5) = 1.75$		
17	.	2	2	2	.	$2.0 + (1/2 \times 0.5) = 2.25$	0.25	2.12
18	1	2	2	.	.	$2.0 + (0 \times 0.5) = 2.00$		
19	.	3	2	.	.	$2.0 + (0 \times 0.5) = 2.00$	0.50	1.75
20	2	3	.	.	.	$1.5 + (0 \times 0.5) = 1.50$		

It should not be asserted that it has now been eliminated as a factor to be considered, and for this reason two tests are still included on each of ten different days before considering an assay complete.

DISCUSSION

The assay value determined as above is but a single point on a dosage-response curve. Several such points are needed, each one for a different strength of extract, before the curve can be reliably defined in full for any drug lot.

It has been the premise that attainment of reasonable homogeneity in test values was a first desideratum in establishment of this method. Attention to date has been concentrated on this aspect of the study. Extension of the assay procedure to include several dosages is now being undertaken.

During the intervening period, a simple assumption has been accepted as a first approximation to the dosage-response curve. Preliminary study had indicated that an approximately rectilinear relationship existed between log concentration and log assay value. An assumption that inverse proportionality existed between strength of extract and assay value within the range of observation of the latter was in order. Whether these linear relationships differ sufficiently for different drug lots to necessitate modification of the initial assumption that they did not, so far as practical consequences of the procedure were concerned, remains to be determined.

One unit[4] was originally established as 0.2 grain of drug lot number 101. This lot had been extensively assayed over a long period of time in a developmental program and its assay value of 2.18, with standard error of 0.103, was fully acceptable as a standard.[5] Other drug lots have been adjusted to this standard in accord with the assumption indicated above. Thus, one unit of D. L. 175 with assay value of 1.87 is given on this theoretical basis by 0.2 (1.87)/2.18 = 0.17 grain. All drug lots are (and have been) thus standardized.

It is not proposed to adhere to the simplifying assumption stated above as a permanent part of the method. Assay procedures usually develop through refinement of cruder precursors. The method reported here is in such process of development. Unique possibilities in bioassay of *Veratrum viride* using *Daphnia magna* are intriguing and have prompted persistence in this study.

It will be noted that the original standard of toxicity, D. L. 101, had a standard error of assay value of 0.103, whereas recent assays (see Table I and reference to D. L. 155) show lower standard errors. This reduction in variability has been achieved along with considerable reduction in number of animals used per assay, from 1,000 for the D. L. 101 standard to 200 routinely at present. Caution is commendable in assigning causes for observed results. Refinements in assay procedure are probably not *entirely* responsible for the increase in homogeneity of results. Continuous selection within the progeny of a few *Daphnia magna* may have contributed materially to this. Indeed it is not at all unreasonable to speculate that the parentage of the present colonies was reduced to a single female at some point.

SUMMARY

1. A method for biologic standardization of *Veratrum viride* has been devised by measuring toxicity of the water-soluble principles on *Daphnia magna*, based on survival time of the test animal.

2. A standard of toxicity has been established in terms of a reference lot of drug which had a toxicity assay value of 2.18 when assayed by the method herein described.

[4] Irwin, Neisler and Co. market the drugs Vertavis and Veratrite standardized in terms of this unit, which is referred to as a "Craw Unit," in this connection.

[5] The original supply of D. L. 101 set aside for assay research is now practically exhausted. A new lot has been extensively assayed in parallel with 101 to establish a new reference standard equivalent to 101.

3. In routine assay procedures the toxicity assay value for each new drug lot is the average of 20 median lengths of life, each of the 20 separate tests involving 10 animals each.

4. Statistical analysis of data indicates that the biologic assay method is highly dependable according to official standards, and results obtained by one technician have been duplicated within entirely acceptable ranges by another.

REFERENCES

(1) Wright, C. R. A., *J. Chem. Soc.*, **35**, 405(1879).
(2) Seiferle, E. J., Johns, I. B., and Richardson, C. H., *J. Econ. Entomol.*, **35**, 35(1942).
(3) Poethke, W., *Arch. Pharm.*, **275**, 357, 571(1937).
(4) Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **155**, 565(1944).
(5) Pilcher, J. D., *Am. J. Physiol.*, **44**, 12(1917).
(6) Pittenger, P. S., *Proc. Penn. Pharm. Assoc.*, 1923.
(7) Rowe, L. W., THIS JOURNAL, **14**, 24(1925).
(8) Christensen, B. V., and McLean, A. P., *ibid.*, **25**, 414 (1936).
(9) Viehoever, A., and Cohen, I., *Am. J. Pharm.*, **111**, 86(1939).
(10) Osol, A., and Farrar, G. E., "U. S. Dispensatory," 24th. ed., J. B. Lippincott, Philadelphia, 1947, pp. 1267–1270.
(11) Viehoever, A., *Am. J. Pharm.*, **109**, 7(1937).
(12) Viehoever, A., and Cohen, I., *ibid.*, **109**, 6(1937).
(13) Viehoever, A., *ibid.*, **109**, 11(1937).
(14) Viehoever, A., *ibid.*, **109**, 12(1937).
(15) Viehoever, A., and Cohen, I., *ibid.*, **110**, 7(1938).
(16) Viehoever, A., and Cohen, I., *ibid.*, **110**, 6(1938).
(17) Viehoever, A., THIS JOURNAL, **27**, 668(1938).
(18) Baylor, E. R., *Biol. Bull.*, **83**, 165(1942).
(19) Obreshkove, V., *ibid.*, **81**, 105(1941).
(20) Noonan, M. J., doctoral thesis on file in the library of Purdue University, 1946.

The Occurrence of a Diphenyl Isatin in California Prunes*

By H. M. BAUM, R. G. SANDERS, and G. J. STRAUB

A commercial water concentrate of California prunes was extracted with chloroform-ethanol solutions which were washed with water to remove dispersed impurities. Evaporation of chloroform revealed a mixture of amorphous and crystalline materials that yielded negative tests for hydroxyanthraquinones and their derivatives, but positive reactions for a diphenyl isatin. A quantitative estimation for diphenyl isatins was devised using di(acetylhydroxyphenyl)isatin as a reference standard based on photometric determinations of color development when diphenyl isatins are treated with sodium hydroxide and potassium ferricyanide solutions.

No specific chemical study of the laxative components in prunes has been made. It was at one time supposed that the cathartic action was due entirely to colloidal and emollient effects in the intestine (1) until Emerson (2) reported the presence of a laxative agent in prunes which had "certain chemical and physiological properties similar to dihydroxy-phenyl isatin and to caffeic and chlorogenic acids." His conclusions are based on the pharmacological activity of various crude fractions on experimental laboratory animals.

It was of interest to determine the chemical nature of the cathartic activity of prunes and to correlate the activity of specific materials with the *in vivo* laxative activity of the prune. This report is accordingly concerned with the separation, identification, and activity of a diphenyl isatin from California prunes.

* Received April 4, 1951, from the Biological Research Division, Harrower Laboratory, St. Louis, Mo.

EXPERIMENTAL

Fractionation.—A water concentrate of California prunes[1] containing 69% solids was used in these studies.

Since the active agents of most of the naturally occurring plant laxatives are fat-soluble hydroxyanthraquinones and their analogs, a chloroform fractionation was decided upon. The prune concentrate was diluted with an equal volume of water and placed in a separatory funnel with four volumes of chloroform and a small amount of ethanol, the latter being effective in preventing emulsification. The chloroform layer was withdrawn and the extraction process was repeated six times using fresh increments of chloroform and ethanol. The chloroform extracts were combined and concentrated to about 10% of the original volume. This concentrate was then washed with water until all traces of dispersed materials were removed (10 washings with equal volumes of water). The material was further concentrated to about 2% of its original volume and placed in an ice chest. Upon cooling, a mixture

[1] Purchased from California Fruit Concentrates, Inc., San Jose, Calif.

of amorphous and crystalline materials was observed. The remaining chloroform was evaporated *in vacuo*.

Chemical Investigation.—Classical qualitative tests for hydroxyanthraquinone derivatives and their analogs were negative (3–5). A qualitative test for diphenyl isatins was found to be positive (6, 7). This reaction is based on color formation when diphenyl isatins are heated with alkali and treated with potassium ferricyanide solution. This color is detectable in dilutions of 1:1,000,000.

Since no reliable quantitative method for diphenyl isatins had been reported,[1] and since a commercial material, di(acetylhydroxyphenyl)isatin was available, it was decided to use this material as a reference standard and the basis for a quantitative method.

Reagents.—(*a*) Di(acetylhydroxyphenyl)isatin (Chemo Puro) stock solution 5 mg./ml. chloroform.

(*b*) 0.01 *N* sodium hydroxide.

(*c*) 5% aqueous potassium ferricyanide (freshly prepared).

(*d*) Alcohol (3A).

Standard Curve.—Amounts of stock solution covering a range of 0.1 to 20 mg. of isatin were placed in test tubes and the chloroform was evaporated from each in a water bath. The residues were then taken up in 20 ml. of alcohol and increased to 50 cc. with distilled water. A blank of 20 ml. of alcohol and 30 ml. of water was prepared. Aliquots of 2.0 ml. were taken from each tube. To these was added 8.0 ml. of sodium hydroxide and 4 drops of potassium ferricyanide solution. The volume of each tube was adjusted to 20 ml. with distilled water and allowed to stand at room temperature for color development. Spectrophotometric readings were taken in a Coleman spectrophotometer at 560 mμ, setting to 100 with the blank as reference solution (see Fig. 1). Because the rate of color development increases with the temperature, readings were taken at five-minute intervals to obtain the point of maximum intensity (fifteen minutes at 25°). It is evident from the graph that the color reaction conforms to Beer's law.

The chloroform fraction of the prune extract showed significant quantities of diphenyl isatin, a total of 200 γ or 1 μg./Gm. of original prune concentrate. It is probable that the original material contained significantly more diphenyl isatin, and that the fractionation was not complete. Absorption curves run over the visible spectrum of the prune extract and synthetic material were identical.

Animal Evaluation.—Animal experiments were conducted using the technique of Macht (8) to compare the laxative activity of the prune concentrate, the chloroform fraction of the prune concentrate, and di(acetylhydroxyphenyl)isatin. Under these conditions it was found that 0.5 Gm. of prune concentrate gave significant response, and 5.0 mg. of di(acetylhydroxyphenyl)isatin and 2.0 mg. of the chloroform extract exhibited definite activity.

[1] Since this research has been completed, a paper by S. Ljunberg in the *Farmaceutisk Revy.*, May, 1950, Stockholm, Sweden, has been published on the photometric determination of acetphenolisatin.

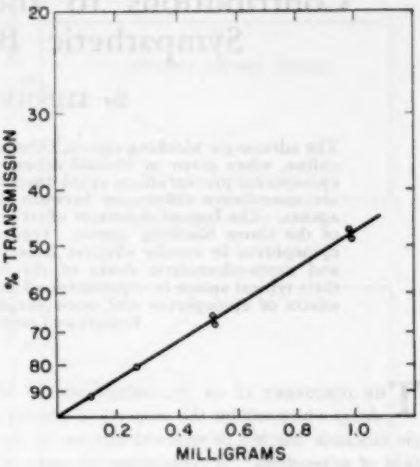


Figure 1

DISCUSSION

The chemical identification of a diphenyl isatin in prune concentrates supports the view that the laxative activity of prunes is due to chemical entities of the water-insoluble intestinal-stimulant type, rather than to colloidal and emollient action. Such materials tend to explain the reported thermostability of the "evacuant principle" of prunes.

Further data, not recorded here, indicate that the diphenyl isatin in prunes is extremely hard to extract; thus these experiments must be regarded as qualitative rather than quantitative as far as the prune fractions are concerned. It is of interest that isatins, including diphenyl derivatives, have been known for some time but have not been heretofore chemically identified as occurring naturally.

SUMMARY

A diphenyl isatin has been identified in a chloroform fraction of an extract of California prunes.

A spectrophotometric method for the quantitative estimation of diphenyl isatin, using synthetic di(acetylhydroxyphenyl)isatin as a standard, is described.

REFERENCES

(1) Sollmann, T., "Manual of Pharmacology," 6th ed., W. B. Saunders Co., 1942, p. 200.
(2) Emerson, G. A., *Proc. Soc. Expil. Biol. Med.*, **31**, 278 (1934).
(3) Warren, L. E., *Am. J. Pharm.*, **44**, 86(1914).
(4) Fuller, H. C., *J. Assoc. Offic. Agr. Chemists*, **9**, 306 (1926).
(5) Fuller, H. C., *ibid.*, **10**, 364(1927).
(6) Baeyer, A., and Lazarus, M. J., *Ber.*, **18**, 2637(1885).
(7) Guggenheim, M., *Schweiz. med. Wochschr.*, **55**, 16 (1925).
(8) Macht, D. I., *Proc. Soc. Expil. Biol. Med.*, **30**, 1272 (1933).

Contributions to the Mode of Action of Sympathetic Blocking Agents*

By THEODORE O. KING†

The adrenergic blocking agents, Dibenamine, yohimbine, benzodioxanes, and Priscoline, when given in divided doses, progressively block epinephrine and nor-epinephrine pressor effects as the doses of the blocking agents are increased. There are quantitative differences between the degrees of efficacy of these blocking agents. The Isuprel depressor effect is enhanced by the administration of several of the above blocking agents. Ergotamine prevents the vasomotor reversal of epinephrine by usually effective doses of the above adrenergic blocking agents, and supra-adrenolytic doses of the blocking agents are necessary to produce their typical action in ergotaminized animals. Ergotamine potentiates the pressor effects of epinephrine and nor-epinephrine and reverses the depressor effects of Isuprel and isopropyl nor-sympatol.

THE DISCOVERY of an increasing number of drugs which exhibit the property of adrenergic blockade has led to renewed interest in the field of adrenolysis. A comparison of many of the pharmacological actions of these compounds has recently appeared (1). The experiments which are reported in this paper represent an attempt to compare both quantitatively and qualitatively the development of blockage induced by five adrenolytic agents under comparable conditions. The pattern of blockade developed by gradually increasing doses of these drugs was studied with respect to the blood-pressure responses caused by small doses of epinephrine, nor-epinephrine, and Isuprel (N-isopropyl-nor-epinephrine). Since the action of epinephrine resembles the combined action of the two latter drugs, the effects of these three drugs were examined to discover if the vasopressor properties of small doses of epinephrine and nor-epinephrine are affected similarly by the adrenolytic agents, and if the vasodepressor properties of epinephrine and Isuprel are correspondingly modified. Quantitative data representing these relationships were obtained and are presented in graphic form.

METHODS

Dogs under pentobarbital sodium (35 mg./Kg.) anesthesia were cannulated for recording mean arterial blood pressure in the usual manner. Control doses of epinephrine, nor-epinephrine, and Isuprel were injected intravenously to produce standard responses of from 20 to 40 mm. Hg change in blood pressure. These doses were generally 0.2 to 0.5 μg./Kg. When reproducible control responses were established, a small dose of the blocking agent was injected intravenously, and, after a suitable period which depended on the agent used, the control doses of epinephrine, nor-epinephrine, and Isuprel were readministered and the effects noted. A second dose of the adrenolytic compound, usually greater than the first, was given and the same procedure followed as before. This experimental sequence was repeated with gradually increasing doses of the adrenolytic agent until sufficient amounts had been administered to produce reversal of the epinephrine pressor response. Quantitative data representing the relationships observed are presented.

RESULTS

Yohimbine Experiments.—Yohimbine hydrochloride, in all doses tested, induced progressive inhibition of the epinephrine pressor responses until these effects were entirely abolished by doses of 0.5 to 1.0 mg./Kg. (Fig. 1 (*A*)). The initial pressor response to epinephrine was progressively reduced by increasing doses of yohimbine above 0.05 mg. The secondary epinephrine depressor response is also reduced in magnitude but rarely is abolished entirely (Fig. 1 (*B*)). With the small control doses of epinephrine, the fall in blood pressure produced following full adrenolytic doses of yohimbine (2.0 mg./Kg.) is usually smaller than the depressor phase of the control response. With larger doses of epinephrine (1.0 μg./Kg.) which normally produce little or no secondary fall in blood pressure, a greater fall is produced after yohimbine.

Nor-epinephrine responses also progressively diminish as the dose of yohimbine is increased. Doses of yohimbine hydrochloride as small as 0.05 mg./Kg. induce 40% inhibition in the nor-epinephrine pressor response (Fig. 1 (*C*)). The greatest degree of inhibition of nor-epinephrine in the doses cited was 90% attained when a total dose level of 12 mg./Kg. yohimbine was reached. Complete abolition of nor-epinephrine pressor effects by yohimbine was not observed.

The depressor response to Isuprel was observed to be either increased or decreased following small doses of yohimbine (Fig. 1 (*D*)). This varied from animal to animal and both phenomena were not seen in the same individual. In two of four animals, inhibition of the Isuprel depressor response followed curves similar to those showing inhibition of the depressor phase of epinephrine (compare upper two curves in Fig. 1 (*B*) with those in 1 (*D*)).

* Received January 24, 1951, from the Department of Pharmacology, Georgetown University, School of Medicine, Washington, D. C.

† Present address: University of Wyoming, College of Pharmacy, Laramie.

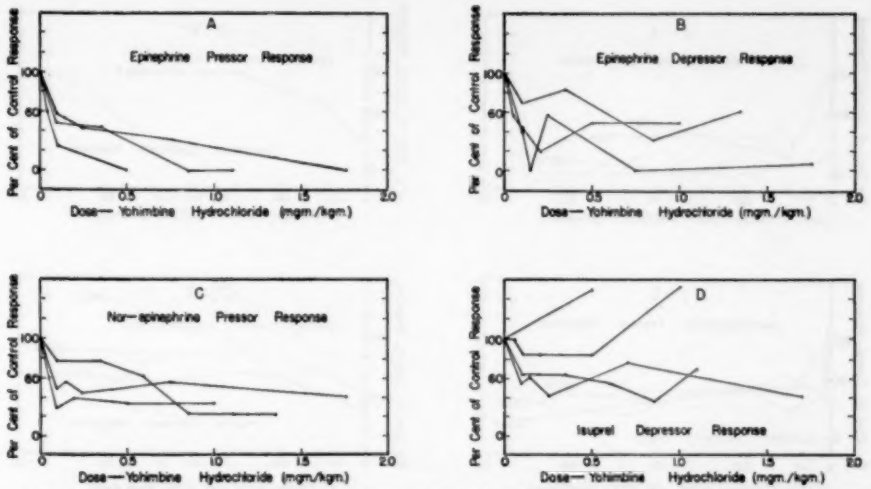


Fig. 1.—Modification of the hemodynamic responses of epinephrine, nor-epinephrine, and Isuprel by increasing the dose level of yohimbine hydrochloride. [Each curve in this and succeeding figures represents results obtained in a single animal.] (Abscissa—concentration of yohimbine hydrochloride in mg./Kg.; ordinate—pressor and depressor effects of epinephrine, nor-epinephrine, and Isuprel, expressed as per cent of control response.)

Benzodioxane Experiments.—The Fourneau compounds, piperidine methyl benzodioxane (933F) and diethylaminomethyl benzodioxane (883F), were employed in this series of experiments. Both compounds produced progressive diminution in the epinephrine pressor response and marked reversal when doses of 1.0 mg./Kg. were reached (Fig. 2 (*A*)). In those animals in which the control dose of epinephrine was biphasic, the fall in blood pressure produced by epinephrine following full adrenolytic doses of 883F and 933F was never of a significantly greater magnitude than the original secondary vasodepression. With small sub-adrenolytic doses of both compounds there is some inhibition of the epinephrine depressor response which disappears as the dose levels of the blocking agents are increased (Fig. 2 (*B*)).

Nor-epinephrine pressor responses were reduced both in height and duration by the Fourneau compounds. Complete abolition of response was never observed with the doses used, more than 50% inhibition rarely being obtained (Fig. 2 (*C*)). In one animal, 883F in doses up to 6.0 mg./Kg. produced no significant reduction in effects of nor-epinephrine, although epinephrine effects were reversed and Isuprel responses characteristically affected.

Isuprel responses are, in general, exaggerated in character by the Fourneau blocking agents (Fig. 2 (*D*)). There is some tendency for the depressor responses to become slightly reduced following small doses of 933F, but after the administration of doses greater than 1.0 mg./Kg. blood-pressure falls as great as two and three times that of the controls have been observed. The occasional initial rise in blood pressure (cardiac effects) caused by Isuprel is also exaggerated by these drugs.

Dibenamine Experiments.—Dibenamine in small doses caused inhibition of both pressor and depressor phases of the control epinephrine response (Fig. 3 (*A*, *B*)). A point was usually reached at a level of 1.0 to 2.0 mg./Kg. Dibenamine hydrochloride at which there appeared a diminution of the Dibenamine inhibition of the epinephrine fall in blood pressure. After this point was reached, the epinephrine fall gradually increased with the increasing dose level of Dibenamine. The pressor phase of the epinephrine response gradually decreased until it disappeared or reached a minimal unalterable point. The "vasomotor reversal" following effective doses (6 mg./Kg. and more) was always of a greater magnitude than the depressor phase of the control responses, and blood-pressure falls two to three times as great as that of the controls were obtained. The duration of fall was often prolonged four to five times.

Diminution, but never complete abolition or reversal, of nor-epinephrine (0.2 to 1.0 μg./Kg.) pressor responses was observed with most doses of Dibenamine. Fifty per cent inhibition of nor-epinephrine responses was observed with 2–5 mg./Kg. Dibenamine hydrochloride. Larger doses exerted only a slighter degree of block (Fig. 3 (*C*)). In one experiment, up to 100% potentiation of nor-epinephrine (0.5 μg./Kg.) responses was observed in doses up to 5 mg./Kg. In this same animal, corresponding potentiation of the pressor phases of the epinephrine and Isuprel responses was also observed.

The Isuprel fall in blood pressure was sensitized by all doses of Dibenamine employed, although maximum potentiation occurred with sub-adrenolytic doses, 1–2 mg./Kg. (Fig. 3 (*D*)). No initial inhibition of response was observed and, depending on the magnitude of the vasodepression produced by the control dose, exaggeration of this response to as much as double the original was obtained.

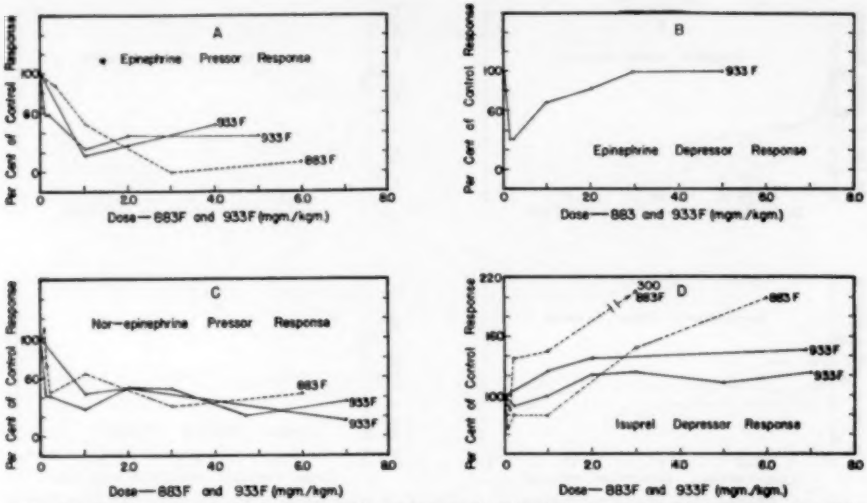


Fig. 2.—Modification of the hemodynamic responses of epinephrine, nor-epinephrine, and Isuprel by increasing the dose level of piperidinomethylbenzodioxane (933F) and diethylaminomethylbenzodioxane (883F). (Abscissa—concentration of 933F and 883F in mg./Kg.; ordinate—pressor and depressor effects of epinephrine, nor-epinephrine, and Isuprel, expressed as per cent of control response.)

Priscoline Experiments.—The depressor phase of the epinephrine control response was completely abolished by Priscoline hydrochloride in doses of 0.5 mg./Kg. and less (Fig. 4 (*B*)). The pressor phase was usually completely blocked by 2.0 to 3.0 mg./Kg. Priscoline hydrochloride (Fig. 4 (*A*)). In one experiment in which 1.0 mg./Kg. Priscoline produced a rise in blood pressure on injection, the hemodynamic responses of epinephrine, nor-epinephrine, and Isuprel were markedly potentiated. Reversal doses not occur until higher dose levels (7–10 mg./Kg.) are reached. The effect obtained with Priscoline is more characteristically an abolition of the epinephrine pressor response than intensification of the depressor effect. The fall in blood pressure produced by epinephrine following adrenolytic doses of Priscoline is usually smaller in depth, although not in duration, than the original depressor

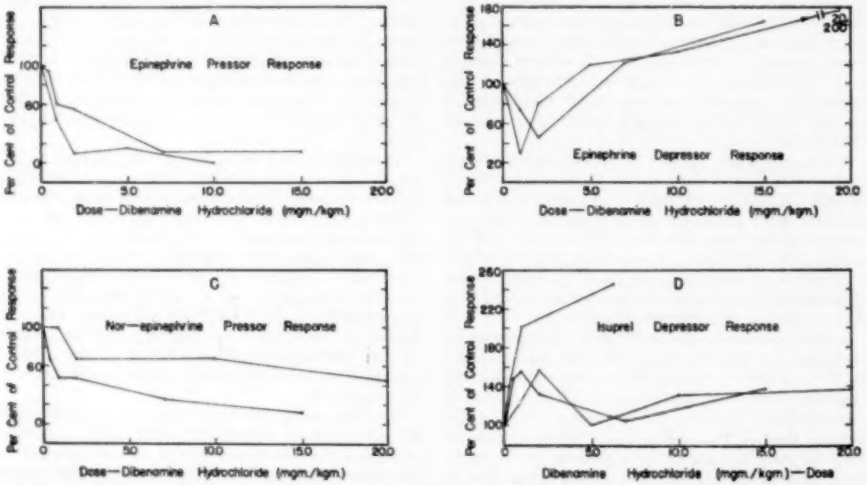


Fig. 3.—Modification of the hemodynamic responses of epinephrine, nor-epinephrine, and Isuprel by increasing the dose level of Dibenamine hydrochloride. (Abscissa—concentration of Dibenamine hydrochloride in mg./Kg.; ordinate—pressor and depressor effects of epinephrine, nor-epinephrine, and Isuprel, expressed as per cent of control response.)

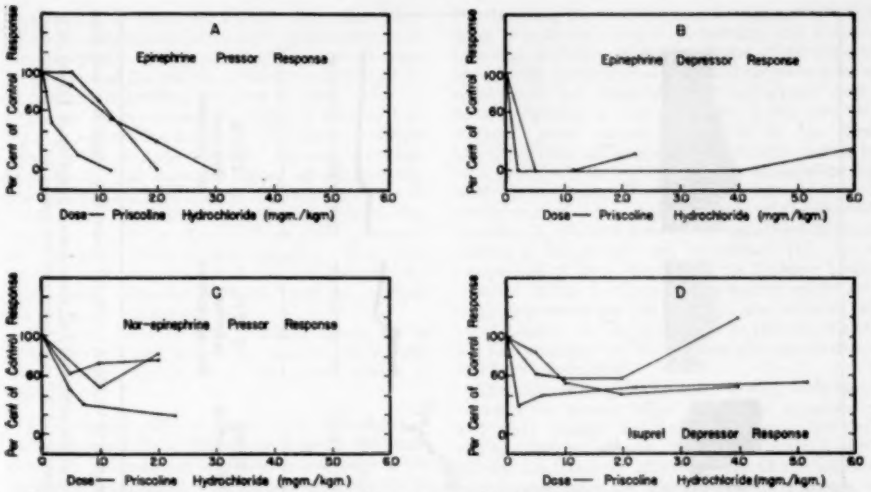


Fig. 4.—Modification of the hemodynamic responses of epinephrine, nor-epinephrine, and Isuprel by increasing the dose level of Priscoline hydrochloride. (Abscissa—concentration of Priscoline hydrochloride in mg./Kg.; ordinate—pressor and depressor effects of epinephrine, nor-epinephrine, and Isuprel, expressed as per cent of control response.)

effect of the small control dose used. Larger doses of epinephrine, however, will produce greater falls in blood pressure.

Nor-epinephrine effects are inhibited by Priscoline in doses as low as 0.2 mg./Kg. (Fig. 4 (*C*)). The extent and rapidity of development of the inhibition of nor-epinephrine varies in the animals studied. However, 50% inhibition of the pressor effects usually appeared following 1.0 mg./Kg. Priscoline. In some animals as much as 100% inhibition was seen after 3.0 mg./Kg. Priscoline, while in others this degree of inhibition was never observed. In one animal in which potentiation of 90% in height was observed, increase in the dose level of Priscoline to 5 mg./Kg. induced typical inhibition of epinephrine and nor-epinephrine responses.

The vasodepression caused by Isuprel is, in most instances, inhibited by Priscoline (Fig. 4 (*D*)). This inhibition produced by Priscoline in doses up to 1.0 mg./Kg. seems to be a mathematical dose-effect relationship. However, when dose levels above 1.0 mg./Kg. are reached, both a constant maintenance of block and decrease in effectiveness of the block have been observed. Larger doses of Priscoline never potentiate the vasodepressor effects of Isuprel.

Ergotamine Experiments.—Ergotamine tartrate, under the conditions of pentobarbital anesthesia employed in these experiments, causes potentiation of epinephrine pressor effects rather than vasomotor reversal (2, 3). In addition, ergotamine (0.025 to 1.0 mg./Kg.) also causes marked potentiation of nor-epinephrine pressor effects and reverses the vasodepressor action of Isuprel into a marked pressor effect (4).

In our experiments, maximum potentiation of the epinephrine pressor response in all experiments was usually manifested following the first dose of ergotamine. The smallest dose of ergotamine employed was 0.025 mg./Kg. Occasionally, some diminution in response to epinephrine was observed as the dosage level of ergotamine was increased. The effects of ergotamine administration on the response to nor-epinephrine follows a similar pattern.

The action of ergotamine on the Isuprel and isopropyl nor-sympatol depressor response is the most singular in this study. Small doses of ergotamine are capable of converting the vasodepressor to vasopressor effects that are generally great in magnitude and duration (Fig. 5). In most experiments it was possible to completely abolish any evidence of the Isuprel fall in blood pressure by increasing the dosage level of ergotamine. In a number of experiments it was not possible to completely abolish a secondary fall in blood pressure, although a strong pressor effect became manifest where none existed in the control response established before ergotamine administration. This increase in blood pressure is not primarily due to an appreciable increase in cardiac acceleration, since the rate of acceleration was seldom more than 10% greater than the control post-ergotamine heart rate. The sympathomimetic nature of the Isuprel reversal seems to be accepted (1). Hazard reported that cocaine can sensitize the Isuprel reversal (5). In our experience this was never observed, although cocaine (10 mg./Kg. intramuscularly) was often administered after reversal had been obtained. The effect of cocaine was not constant, although generally it seemed to reduce the pressor effect or to induce the re-emergence of the depressor response to Isuprel. At the same time, however, cocaine usually caused an increase in the potentiated pressor effects of epinephrine and nor-epinephrine.

The action of ergotamine in establishing the above effects in the presence of blocking concentrations of adrenolytic agents have been presented in a previous paper (6). In addition, it has also been

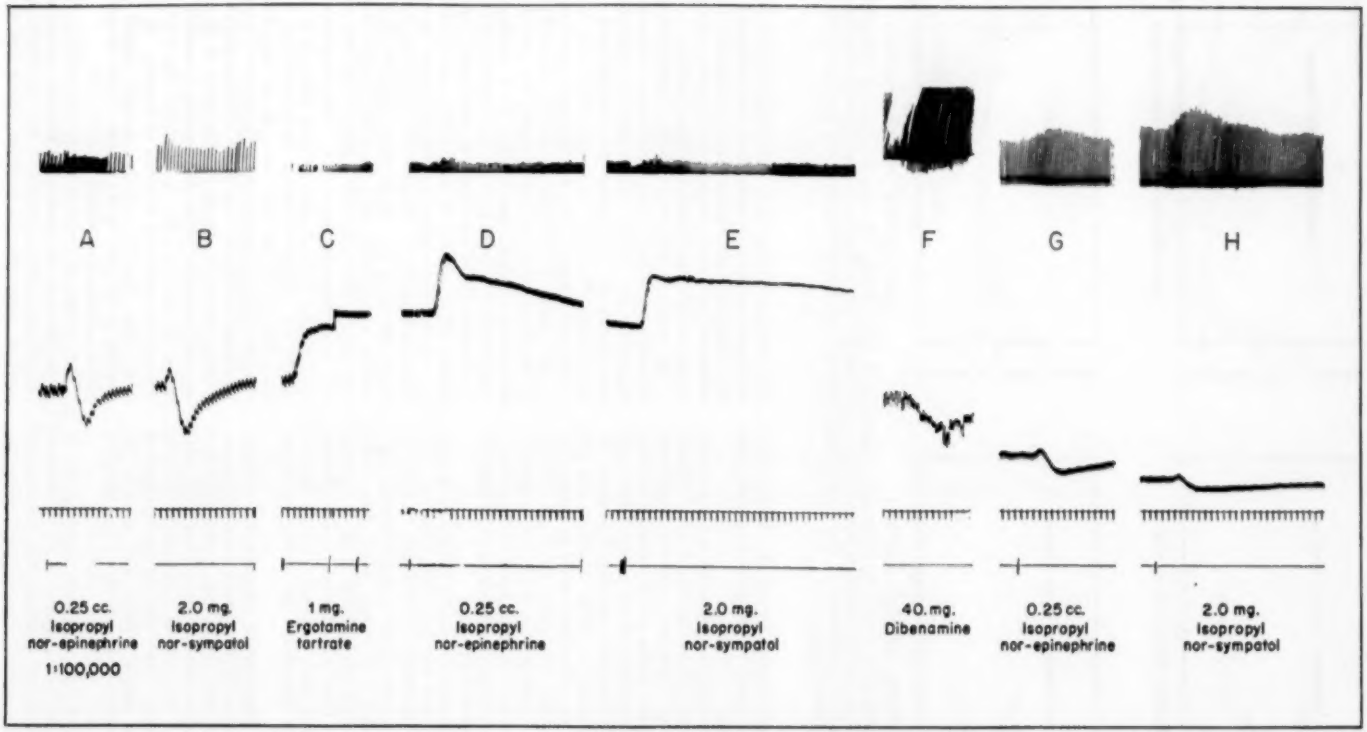


Fig. 5.—Dog, male, 19.0 Kg.; pentobarbital sodium anesthesia. Upper line—respiration; second line—mean arterial blood pressure; third line—base line and time signal (five seconds); bottom line—injection marks.

A, intravenous injection of 0.25 cc. Isuprel (1:100,000) total dose; *B*, intravenous injection of 2.0 mg. isopropyl nor-sympatol; *C*, intravenous injection of 1.0 mg./Kg. ergotamine tartrate; *D*, intravenous injection of 0.25 cc. Isuprel (1:100,000) total dose; *E*, intravenous injection of 2.0 mg. isopropyl nor-sympatol; *F*, intravenous injection of 40.0 mg./Kg. Dibenamine hydrochloride; *G*, as under *A*; *H*, as under *B*.

found that in order to reverse the ergotamine-induced modification of Isuprel, epinephrine, and nor-epinephrine effects, adrenolytic agents in doses far in excess of the normal adrenolytic dose must be employed. Otherwise, no effect is noted. For example, in an experiment in which a total of 0.95 mg./Kg. ergotamine had been administered, pressor effects of 40, 44, and 100 mm. Hg were recorded, respectively, for standard doses of epinephrine, nor-epinephrine, and Isuprel. Increasing doses of Dibenamine hydrochloride, until a total concentration level of 11.5 mg./Kg. had been given, produced no diminution in the pressor responses. In general 20–40 mg./Kg. of Dibenamine was necessary to induce usual adrenolytic effects, and to produce Isuprel vasodepression (Fig. 5).

Similarly, doses of other blocking agents far in excess of those which normally produce adrenolysis were required to counteract the above effects of ergotamine.

Experiments with Cocaine and Ephedrine.—To further elucidate differences between the vasodepressor properties of epinephrine and Isuprel, the effects of cocaine (10 mg./Kg. intramuscularly) on vasomotor reversal and on Isuprel were studied. It was found that while cocaine was capable of accentuating the normal vasodepression caused by Isuprel, it had either no effect on vasomotor reversal or it had an inhibitory effect. In no case was reversal potentiated by intramuscularly injected cocaine. In an experiment in which Dibenamine was used to induce reversal, cocaine had no effect and in experiments utilizing Priscoline and yohimbine as the blocking agents, vasodepression was greatly decreased or abolished. In both the latter cases complete reversal was restored by injection of additional doses of the adrenolytic agent.

Ephedrine sulfate (4 mg./Kg.) was also found to potentiate the normal Isuprel response in addition to epinephrine and nor-epinephrine effects. When cocaine hydrochloride (10 mg./Kg.) was administered intramuscularly in an animal in which potentiation of the normal effects of the three amines had been produced by ephedrine, the responses to epinephrine and nor-epinephrine were potentiated still further. *When Isuprel was injected, however, instead of a correlative synergistic response obtaining, there followed a rise in blood pressure from this inhibitory amine.* This rise was reproducible for an hour, after which the depressor effects began to return as part of a diphasic response. During this time the potentiated epinephrine response also gradually diminished in magnitude. After two and one-half hours, an additional intramuscular injection of cocaine hydrochloride (10 mg./Kg.) failed to restore a pure pressor effect, but an additional dose of ephedrine (2.0 mg./Kg.) restored the purely pressor effect of the isopropyl analog.

DISCUSSION

In the experiments which have been presented, an attempt has been made to study the pattern of adrenolysis induced by a number of adrenolytic agents. The five adrenolytic agents, yohimbine, 883F, 933F, Dibenamine, and Priscoline, differ from ergotamine in being capable of producing epinephrine reversal regardless of the anesthetic use. By observing the modifications in response to small doses of epinephrine, nor-epinephrine, and Isuprel, following gradually increasing doses of the adrenolytic agents, a differential picture of the properties of epinephrine and those of pharmacologically simulated sympathin E and sympathin I has been developed. Since natural sympathin E has been identified with nor-epinephrine, recently shown to be a product of sympathetic nerve stimulation by Bülbring and Burn (7) and by West (8), and to be a constituent of the natural secretion of the adrenal medulla (9, 10), and since the sympathin-I-mimetic actions of Isuprel are well established (11, 12), a comparison of these effects with the biphasic hemodynamic E and I effects of epinephrine elicits differences between the two E effects and between the two I effects. At the same time an effective standard for comparison of the various adrenolytic drugs has been established.

The experiments demonstrate several differences between the pressor effect caused by epinephrine and that caused by nor-epinephrine. Whereas the epinephrine pressor effect is, in general, completely abolished by effective doses of the adrenolytic compounds, the nor-epinephrine response is rarely more than 80% inhibited in magnitude and is never reversed. The inhibition of nor-epinephrine follows that of epinephrine very closely in relation to the dose of the adrenolytic agent. But, when the point of maximum diminution in response to nor-epinephrine (rarely 100%) is obtained, increasing doses of the adrenolytics will completely abolish the pressor epinephrine effect.

As the observed epinephrine pressor effect is believed to represent the resultant of the effects of sympathetic excitatory and inhibitory mechanisms, it was expected that with progressive block of the pressor phase, a corresponding deepening of the fall of blood pressure would occur. This, however, was never seen with any of the adrenolytic agents used. In fact, the opposite took place. With small doses of all the agents a decrease in the epinephrine depressor response occurred, together with the diminution of the epinephrine pressor effect. With constant small doses of epinephrine which normally produced a biphasic vasomotor response, the magnitude of the vasomotor reversal, *except in the case of Dibenamine*, was never greater than the original depressor phase of the control epinephrine injection. Following Dibenamine, however, progressive sensitization of the epinephrine fall in blood pressure was obtained with increasing doses of the adrenolytic agent. This sensitization followed an initial inhibition of the depressor response by small doses which was similar to that obtained with Priscol, the Fourneau compounds, and with yohimbine. The epinephrine fall in blood pressure was increased as much as 100% by doses of Dibenamine from 10 to 20 mg./Kg. Only the Fourneau compounds show a somewhat similar effect in producing vasomotor reversal. Following initial inhibition, the fall in blood pressure gradually increases but rarely does it finally become greater than the depressor phase of the control response.

These experiments show that the sensitivity of the vasodilator mechanisms also decreases following the administration of adrenolytic agents. As the sensitivity of the sympathetic vasodilator elements can be demonstrated with very small doses of

epinephrine, it would appear that the fall in blood pressure following adrenolysis may be different from the normal depressor phase of epinephrine action. A similar suggestion has recently been made by Freyburger, Capo, and Moe (13), based on somewhat different evidence. In addition, these experiments demonstrate that Dibenamine, and possibly the Fourneau compounds, owe much of their adrenolytic action to the *sensitization* of the vasodilator mechanisms. This corresponds to the explanation of ergotamine action in producing vasomotor reversal given by Herwick, Linegar, and Koppanyi (3). However, Priscoline and yohimbine do not appear to possess this property. It might also be postulated that the latter two drugs may injure the vasodilator apparatus, rendering them less responsive to epinephrine stimulation.

The effects of the adrenolytic compounds on the action of the sympathin-I-mimetic compound, Isuprel, seem to parallel their effects on the depressor phase of epinephrine. Both Dibenamine and the Fourneau compounds sensitize the fall in blood pressure due to Isuprel. It is interesting that small doses of Dibenamine which exert very slight or no effect on epinephrine responses are sufficient to sensitize the vasodepressor effects of Isuprel. This does not occur with the other agents used. Yohimbine has been observed to inhibit the action of Isuprel in some animals and to potentiate it in others. Priscoline action is usually inhibitory. A close correlation is thus observed between the sympathin-I-like action of Isuprel and that of epinephrine in relation to adrenolytic compounds.

The sensitization of the epinephrine and nor-epinephrine pressor effects by ergotamine under pentobarbital anesthesia may be very much like that of cocaine. In both cases the pressor effect is potentiated both in length and duration. The post-ergotamine response is almost always greater than that following 5–10 mg./Kg. cocaine. Correlative observations have also been made regarding the effects of cocaine and ergotamine in potentiating the sympathiomimetic pressor actions of acetaldehyde and acetaldehyde ammonia (14, 15). That the two alkaloids do not attack a single common mechanism is shown by their opposite effects on Isuprel action. Cocaine induces potentiation and ergotamine reversal. However, the ergotamine reversal of Isuprel can be duplicated in the cocainized animal by the supplementary administration of ephedrine. Ephedrine, as is well known, also potentiates epinephrine pressor effects (16, 17). In addition, ephedrine alone potentiates the normal effects of Isuprel as does cocaine. It would appear, therefore, that cocaine and ergotamine affect some common mechanism with respect to the sensitization of the pressor effects of these compounds. Since ephedrine converts the potentiation of Isuprel by cocaine to a reversal, it also seems that the ephedrine-cocaine combination modifies the same factors as ergotamine in producing this effect. It remains to be investigated whether or not other ergotamine effects can be duplicated by combined cocaine-ephedrine administration.

SUMMARY AND CONCLUSION

The adrenolytic compounds examined progressively block epinephrine and nor-epinephrine pressor responses as the doses of the blocking agents are increased.

The vasomotor reversal produced following adrenolytic doses of Dibenamine, dimethylaminomethylbenzodioxane, and piperidinomethylbenzodioxane by constant doses of epinephrine increases as the dose of the blocking agent is increased. The magnitude of reversal following these three drugs is usually greater than the depressor component of the control dose of epinephrine. The vasomotor reversal following effective doses of the adrenolytic agents increases with increasing doses of epinephrine. The Isuprel depressor effect is also sensitized by these three drugs. Similarity is thus shown between the two inhibitory responses.

The vasomotor reversal produced by small doses of epinephrine following Priscoline is usually not so deep as that produced by similar doses of epinephrine after Dibenamine and the Fourneau compounds. The effect of yohimbine is intermediate between Priscoline and the latter drugs.

The adrenolytic actions of Dibenamine, the Fourneau compounds, and perhaps yohimbine are possibly due to sensitization of the vasodilator sympathetic elements. Priscoline does not appear to produce sensitization of the vasodilator mechanisms.

Isuprel vasodepression and the inhibitory vasomotor actions of epinephrine are similar in relation to the adrenolytic compounds and differ only in their reaction to cocaine.

Supra-adrenolytic doses of the blocking agents are necessary to produce their typical actions in the ergotaminized animal.

Ergotamine potentiates the pressor actions of epinephrine and nor-epinephrine, and reverses the depressor effects of Isuprel.

Both ephedrine and cocaine potentiate the depressor actions of Isuprel.

Ephedrine and cocaine in the same animal reverse the effects of Isuprel, while potentiating the pressor effects of epinephrine and nor-epinephrine.

The relationship of epinephrine reversal to the theory of epinephrine action is discussed in this paper.

REFERENCES

(1) Nickerson, M., *J. Pharmacol. Expll. Therap.*, **95**, Part II, 27(1949).
(2) Herwick, R. P., Linegar, C. R., and Koppanyi, T., *ibid.*, **63**, 15(1938).
(3) Herwick, R. P., Linegar, C. R., and Koppanyi, T., *ibid.*, **65**, 185(1939).
(4) King, T. O., *Federation Proc.*, **8**, 308(1949).
(5) Hazard, R., Beauvallet, M., and Giudicelli, R., *Compt. rend. soc. biol.*, **142**, 189(1948).
(6) King, T. O., and Koppanyi, T., THIS JOURNAL, **38**, 346(1949).
(7) Bülbring, E., and Burn, J. H., *Nature*, **163**, 363 (1949).
(8) West, G. B., *ibid.*, **163**, 721(1949).
(9) Holton, P., *ibid.*, **163**, 217(1949).
(10) Goldenberg, M., Faber, M., Alston, E. J., and Chargaff, E. C., *Science*, **109**, 534(1949).
(11) Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M., *J. Pharmacol. Expll. Therap.* **92**, 369(1948).
(12) Lands, A. M., Rickard, E. B., Nash, V. L., and Hooper, K. E., *ibid.*, **89**, 297(1947).
(13) Freyburger, W. A., Capo, L. R., and Moe, G. K., *Federation Proc.*, **8**, 293(1949).
(14) Koppanyi, T., *Anesthesiology*, **6**, 603(1945).
(15) Koppanyi, T., *Federation Proc.*, **4**, 124(1945).
(16) Koppanyi, T., and Luckhardt, A. B., *Arch. intern. Pharmacodynamie*, **40**, 344(1931).
(17) Burn, J. H., *J. Pharmacol. Expll. Therap.*, **46**, 75 (1932).

Determination of *Gamma*-1,2,3,4,5,6-Hexachlorocyclohexane in Lotions and Ointments*

By JOHN H. FOSSUM and JOHN A. RIDDICK

A method is presented for the polarographic determination of γ-1,2,3,4,5,6-hexachlorocyclohexane in lotions and ointments containing 0.5 or 1.0 per cent of the active ingredient. The method is rapid and has an accuracy of ±2 per cent or better.

THE polarographic reduction of γ-1,2,3,4,5,6-hexachlorocyclohexane (commonly referred to as γ-benzene hexachloride) has been reported by a number of investigators (2–5). These methods cannot be applied directly to the analysis of lotions and ointments because components of the base interfere.

A study of the polarographic reduction of γ-benzene hexachloride carried out in these laboratories has shown that the shape of the wave can be altered by changing the base solution. One long wave is obtained using gelatin as maximum suppressor (2); two distinct waves can be obtained with several other maximum suppressors. The base constituents of the lotions and ointments tested did not interfere with the measurement of the first wave, so the height of this wave was measured to determine the concentration of γ-benzene hexachloride.

The first wave shows a large maximum which is difficult to suppress. The only compounds of those tested which would suppress this maximum were 0.0004 per cent acid fuchsin, 0.001 per cent *p*-sulfo-*o*-methoxybenzeneazodimethyl-1-naphthylamine, and 0.001 per cent basic fuchsin. However, the solutions of *p*-sulfo-*o*-methoxybenzeneazodimethyl - 1 - naphthylamine and basic fuchsin were not effective until they had been allowed to age for six months to one year. The change that these compounds undergo during the aging of their solutions is apparently neither oxidation nor reduction because electrolytic oxidation or reduction of these solutions failed to yield a solution which would suppress the maximum of the first wave. Further investigation of this phenomenon is contemplated.

Best results were obtained with a base solution containing 0.1 *M* tetramethylammonium bromide and 0.0004 per cent acid fuchsin in methanol. The half-wave potentials of the two waves are −1.146 volts and −1.395 volts measured against the mercury pool. The height of the first wave is approximately twice that of the second wave.

The preliminary investigation was carried out using a Sargent Model XXI Polarograph; however, a Sargent Model III Polarograph is used in these laboratories for routine determinations.

EXPERIMENTAL

Apparatus and Reagents

(*a*) Polarograph, Sargent Model III and accessories.

(*b*) Electrolysis vessel, Sargent Catalog No. S-29307.

* Received May 31, 1951, from the Research and Development Department, Commercial Solvents Corporation, Terre Haute, Ind.

(*c*) γ-Benzene hexachloride, purity at least 99.9% minimum m. p. = 112.79°, as determined by the method of Toops and Riddick (6).

(*d*) Methanol, A. C. S. reagent grade.

(*e*) Tetramethylammonium bromide, Eastman Kodak Co., highest purity.

(*f*) Maximum suppressor, stock solution: dissolve 100 mg. of acid fuchsin in methanol and dilute to 100 ml. Dilute 10 ml. of this stock solution to 100 ml. with methanol for use.

(*g*) Mercury, polarographically pure.

(*h*) Nitrogen, oxygen-free.

PROCEDURE

Determination of Diffusion Current Constant

Accurately weigh 30–40 mg. of standard γ-benzene hexachloride into a tared 25-ml. volumetric flask. Dilute to volume with methanol and mix thoroughly. Pipette 5.00 ml. of this solution into a clean 25-ml. volumetric flask, add 0.3852 Gm. of tetramethylammonium bromide, 1.00 ml. of maximum suppressor, and 10 ml. of methanol. Warm on the steam bath until dissolved, dilute the solution to slightly less than 25 ml. with methanol, and mix. Place the flask in a thermostat at 25.0 ± 0.1° for at least fifteen minutes, dilute the solution to volume with methanol, and mix thoroughly.

Make two prebubblers from 50-ml. conical-type centrifuge tubes. Put about 10–15 ml. of methanol in the first prebubbler and about 10 ml. of the solution to be analyzed in the second. This will minimize change in concentration due to evaporation of methanol during deaeration.

Transfer the remainder of the solution to be analyzed to the electrolysis vessel thermostated at 25.0 ± 0.1°. Bubble nitrogen through the solution for nine minutes. Add mercury to the cell for the anode, insert the dropping mercury capillary, and allow nitrogen to bubble through the solution for an additional three minutes. Stop the flow of nitrogen and determine the drop time. Measure the diffusion current by reading the maximum galvanometer deflection at −1.300 volts and at −0.600 volts after the galvanometer has attained equilibrium at each voltage.

Calculation

$$J = (i_h - i_l) \times s \times k_c \times 0.03075/w$$

in which

J = corrected diffusion current constant in microamperes per mg. γ-benzene hexachloride.
i_h = galvanometer reading at −1.300 volts.
i_l = galvanometer reading at −0.600 volt.
s = setting of current multiplier.
w = mg. of standard γ-benzene hexachloride in original solution.
$k_c = 1/m^{2/3}t^{1/6}$

where

m = rate of flow of mercury through the capillary in mg. per sec.
t = drop time in sec.

The numerical constant is the galvanometer sensitivity of the polarograph multiplied by the dilution factor.

Analysis of Sample

Accurately weigh approximately 0.5 Gm. of a lotion or ointment containing 1.0% γ-benzene hexachloride or 1.0 Gm. of a lotion or ointment containing 0.5% γ-benzene hexachloride into a tared 25-ml. volumetric flask. (Lotions can be conveniently transferred with a 1-ml. pipette, and ointments can be conveniently transferred with a piece of 6-mm. glass tubing.) Add 0.3852 Gm. of tetramethylammonium bromide, 1.00 ml. of maximum suppressor, and 15 ml. of methanol. Warm on the steam bath until dissolved, dilute the solution to slightly less than 25 ml. with methanol, and mix. Place the flask in a thermostat at 25.0 ± 0.1° for at least fifteen minutes. Dilute the solution to volume with methanol and mix thoroughly. Deaerate the solution and measure the wave height as described under "Determination of Diffusion Current Constant."

Calculation

$$\%\gamma = (i_h - i_l) \times s \times k_c \times 0.615/J \times w$$

in which w = wt. of sample in mg.

The other symbols have the same significance as described above. The numerical constant is the sensitivity of the galvanometer on the polarograph multiplied by 100.

RESULTS

The accuracy of the method was determined by preparing Kwell[1] lotion containing 1.00% standard γ-benzene hexachloride. The following results are typical of those obtained for the analysis of this sample: 1.01, 1.02, 0.98, 0.98, 0.99, mean = 1.00%, mean deviation = 0.016. The 95% confidence interval (1) is 1.00 ± 0.020. Similar precision can be obtained with production-run ointments and otions.

LIMITATIONS

None of the lotions and ointments prepared in these laboratories gave any interference with this method. Before the method is used for any similar lotions or ointments, a complete curve of the base formula without the active ingredient should be plotted to determine whether any of the constituents interfere.

REFERENCES

(1) Dean, R. B., and Dixon, N. J., *Anal. Chem.*, **23**, 636 (1951).
(2) Dragt, G., *ibid.*, **20**, 737(1948).
(3) Ingram, G. B., and Southern, H. K., *Nature*, **161**, 437 (1948).
(4) Katumura, Y., Suzuki, M., Jakajima, M., and Okubo, T., *Botyu-Kagaku* (*Sci. Insect Control*), **11**, 3(1949).
(5) Schwabe, K., *Z. Naturforsch.*, **3**, 217(1948).
(6) Toops, E. E., and Riddick, J. A., *Anal. Chem.*, in press.

[1] Trade mark of Commercial Solvents Corporation.

Assay of Pharmaceutical Tablets Containing Calcium by the Schwarzenbach Method*

By GEORGE B. GRIFFENHAGEN, JOAN L. PFISTERER, and SELMA K. SLOTH

The Schwarzenbach method has been applied to a series of tablets containing calcium. The method consists essentially of titrating calcium in solution with disodium ethylenediamine tetraacetic acid (ETA) in the presence of a small amount of magnesium, and using eriochrome Black-T as indicator. The accuracy of the method is practically the same as the official oxalate method with which it has been compared. The ETA method, however, effects a considerable saving of time, which is its main advantage over the oxalate method.

A REVIEW of the Schwarzenbach method for the determination of calcium and an investigation of its applications to pharmaceutical products containing calcium has been made by Mattocks and Hernandez (1). Initial investigations have indicated that the method is applicable to the following N. F. salts: calcium bromide, calcium levulinate, calcium hypophosphite, calcium glycerophosphate, and lime. Subsequent work by Hernandez, *et al.* (2), indicates that the Schwarzenbach method is also applicable to other calcium salts as well as many official magnesium and mercury compounds.

The convenience and accuracy of the ETA method has led the authors of this paper to investigate its application to pharmaceutical tablets containing calcium. A series of sample compressed tablets containing calcium in the form of calcium gluconate, dibasic calcium phosphate, tribasic calcium phosphate, calcium lactate, bone calcium, and calcium succinate in combination with most common minerals and vitamins used in medicine were assayed by both the official oxalate method and the ETA method.

EXPERIMENTAL

The oxalate method used on all samples was the same procedure as directed in the *Assay* under *Precipitated Calcium Carbonate*, U. S. P. XIV (3). The ETA method was similar to that method proposed by Mattocks and Hernandez (1), with the exception that double-strength ETA reagent and magnesium chloride solution were used, permitting the use of smaller volumes of the standard solutions. The procedure in detail is as follows:

ETA Reagent.—Dissolve about 20 Gm. of disodium ethylenediamine tetraacetic acid in sufficient water to make 1 L.

Magnesium Chloride Solution.—Dissolve 15 Gm. of reagent magnesium chloride in sufficient water to make 1 L.

Buffer Solution.—Dissolve 67.5 Gm. of ammonium chloride and 570 cc. of concentrated ammonium hydroxide in enough water to make 1 L.

Indicator Solution.—Dissolve 5 Gm. of ManVer indicator powder (Hach Chemical Company, Ames, Iowa), consisting of 1 part of eriochrome Black-T and 9 parts of hydroxylamine hydrochloride, in sufficient methanol to make 100 cc.

Standardization of Magnesium Chloride Solution.—Transfer 25 cc. of ETA solution to a 250-cc. flask, add four drops indicator solution and 5 cc. buffer solution and titrate with magnesium chloride solution to a wine red. Calculate the ratio (R) as cc ETA/cc. $MgCl_2$.

Standardization of ETA Solution.—Weigh 100 mg. of reagent calcium carbonate into a 250-cc. flask. Dissolve in 25 cc. of water and 10–15 drops of dilute hydrochloric acid. Add 25 cc. of ETA solution accurately measured, four drops of indicator solution, and 5 cc. of buffer solution. Titrate with magnesium chloride solution to a wine red Each cubic centimeter of ETA solution is equal to:

$$\frac{\text{mg. } CaCO_3 \times 0.4}{\text{cc. ETA} - (\text{cc. } MgCl_2 \times R)} \text{ mg. calcium}$$

Titration of Calcium Salts in Pharmaceutical Tablets.—An amount of calcium mixture equivalent to about 30–40 mg. of calcium is weighed into a 250-cc. flask and is dissolved in 10–15 drops of dilute hydrochloric acid. Fifty cubic centimeters of water, 25 cc. of ETA solution accurately measured, six drops of indicator solution, and 5 cc. of buffer solution are added in that order, and the solution is titrated with magnesium chloride solution to a wine red. The number of milligrams of calcium per tablet is calculated as follows:

$$\frac{[\text{cc. ETA} - (\text{cc. } MgCl_2 \times R)] \times \text{ETA Ca factor}}{\text{number of tablets taken}} = \text{mg. Ca/tablet}$$

DISCUSSION

Only in the case of multivitamin tablets using magnesium oxide to absorb the A, D, and E oils was any special consideration required using the ETA method. In the presence of magnesium oxide, the calcium content was calculated as follows:

$$\text{Ca determined} - (\text{wt. MgO/tab.} \times 0.6032 \times 1.67) = \text{Ca present}$$

Magnesium stearate when used as a lubricant made no significant difference in the results, eliminating the necessity of any factor.

* Received December 19, 1950, from the Analytical Laboratory of Neoco Corporation, Los Angeles, Calif.

TABLE I

Sample No.	Type Tablet	Form of Calcium	Label Claim, Mg.	Average of Duplicate Samples, Mg. Oxalate Method	ETA Method
1	Vitamins and calcium	A	62.5	72.4	73.1
2	Vitamins and calcium	A	62.5	78.0	75.2
3	Multivitamin	A & B	116.7	121.1	127.6
4	Calcium tablet	C & D	79.0	89.4	86.2
5	Calcium wafer	A & C	165.0	171.3	171.8
6	Mineral tablet	A & E	125.0	132.2	130.4
7	Calcium tablet	C & D	79.0	87.5	86.2
8	Calcium tablet	A & C	85.0	89.1	87.9
9	Vitamins and calcium	A	62.5	75.9	75.4
10	Calcium wafer	A & C	165.0	176.1	175.8
11	Calcium wafer	A & C	165.0	177.8	179.0
12	Vitamins and calcium	A	62.5	80.5	79.9
13	Vitamins and calcium	A	62.5	71.5	74.1
14	Multivitamin	A & G	104.0	111.4	111.6
15	Multivitamin	A & G	64.8	73.9	74.0
16	Vitamins and calcium	A	62.5	79.3	73.9
17	Mineral tablet	B	158.0	176.2	163.9
18	Vitamins and calcium	A	62.5	79.3	75.6
19	Calcium with iron	A & C	85.0	86.4	94.8
20	Multivitamin	A, B & G	116.7	117.3	123.9
21	Calcium succinate	F	29.8	31.6	31.4
22	Multivitamin	A & G	116.0	139.3	143.4
23	Calcium tablet	B	250.0	257.2	260.0
24	Mineral tablet	B	158.0	173.9	167.2
25	Mineral tablet	B	160.0	193.6	186.3
26	Calcium tablet	C & D	79.0	88.8	83.4
27	Vitamins and calcium	A	62.5	75.8	74.6
28	Mineral tablet	A & E	125.0	130.5	135.9

A = Dicalcium phosphate. B = Tricalcium phosphate. C = Calcium gluconate. D = Calcium lactate. E = Bone calcium. F = Calcium succinate. G = Magnesium oxide (factor used to compensate for Mg content).

CONCLUSIONS

There is no indication that there is any difference in the accuracy of the ETA method as compared with the official oxalate method on pharmaceutical tablets containing various calcium salts in combination with most common minerals and vitamins used in medicine. The ETA method requires approximately fifteen minutes for the assay of a pair of samples, while the official oxalate method requires three to three and one-half hours. Thus the ETA method represents a considerable saving of time over the official oxalate method.

REFERENCES

(1) Mattocks, A. M., and Hernandez, H. R., THIS JOURNAL, **40**, 519(1950).
(2) Hernandez, H. R., Biermacher, U., and Mattocks, A. M., *Bull. Natl. Formulary Comm.*, **17**, 145(1950).
(3) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950.

☆

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Brassidic acid
2,2′-Biquinoline
8-Hydroxycoumarin
Nethamin hydrochloride
Fabiatrin
Scopolin
Tibatin
2,3-Angelica lactone
Desoxyribose
1,2-Diketohydrindene
Trilauryl hypophosphate
Sodium myristyl sulfate
Methyl erucate
Magnesium saccharate
Aluminum methionate
1,4-Diaminopentane
Anthrylmercapturic acid
Uramildiacetic acid
Toxisterol
Thymidylic acid

Book Reviews

Paracelsus (*Magic into Science*). By HENRY M. PACHTER. Henry Schuman, Inc. New York, 1951. x + 360 pp. Illustrated. 14.5 x 21.8 cm. Price $4.

The name "Paracelsus" is known to chemists mainly because he was responsible, in part, for freeing chemistry from the superstitions of alchemy. Because Paracelsus' approach to chemistry was through medicine and pharmacy, his name is also well known to those familiar with the medical and allied sciences. When the name "Paracelsus" is mentioned, it usually recalls to mind that he taught that the object of chemistry is not to make gold but to prepare medicines, and that he regarded the healthy human body as a combination of chemicals, the imbalance of which resulted in illnesses that could be cured by means of administering the right chemical. He was thus the first to challenge the principles of Galen and may now, in retrospect, be credited with having envisioned the great advances in chemotherapy as we know them today.

Paracelsus is remembered, even by the most superficial student of pharmaceutical and chemical history, for having been one of the first to employ copper, lead, antimony, and other inorganic compounds as medicines. In this way he stimulated the development of the profession of pharmacy by adding many drugs to the materia medica of the 16th century. One historian considers that "pharmacy owes everything to Paracelsus." He doubtless created an interest in chemistry in the minds of physicians and pharmacists of his time, which resulted in the development of that science.

Much has been written about Paracelsus, not only by students of the history of science, but also by imaginative writers. Dr. Pachter, author of the most recent work on Paracelsus, has prepared a most worthy addition to the *Life of Science Library*, published by Henry Schuman, Inc. The author considers the story of the life of Paracelsus as a clue to the riddle of the Renaissance and develops his subject from that standpoint in a most interesting manner. He considers that Paracelsus, quite apart from his special significance in science history, ranks with the most representative figures of the age of the Renaissance and the Reformation. Even a cursory reading of the book impresses one with the versatility of the mind and interests of Paracelsus extending far beyond the confines of chemistry, medicine, or pharmacy.

The book is written in a style that has appeal both to the casual reader and to the student of history. It is provided with notes in which sources of direct quotations are given, and a series of appendixes in which additional notes interpreting Paracelsus to the reader are offered and other interpretive material is presented. The book is written in a style intended to stimulate and hold the interest of the reader, as well as to impart information about a great and contradictory historical character and the time during which he lived. The first paragraph of the book reads as follows:

"In the year of our Lord, one thousand five hundred and twenty-five—not long after Columbus had landed in the West Indies—a square-built man of odd appearance rode into the Emperor's good town of Ingolstadt on the Danube. His satchel was bursting with medicine pots, precious stones, astrological charts, and surgical instruments. His hat and coat showed the lining of miniver fur doctors were privileged to wear, but from his belt hung an enormous sword, and his leather jacket was dusty and sooty. People noticed that the stranger wore his sword wherever he went, and the wiseacres whispered: 'It's bewitched; in the pommel he keeps the Elixir of Life.' "

The above paragraph is quoted to illustrate the vivid style used by the author throughout the book, whereby Paracelsus is brought into sharp focus and made to seem quite real. In Paracelsus one sees the counterpart of men now living, and the story of the world of his time in its struggles of ideologies, fanatical faith, and its intellectual differences, is reminiscent of the present. Throughout the 26 chapters of the book, the life of Paracelsus and the history of his time are portrayed in a fascinating manner.

Mechanism and Evaluation of Antiseptics. Annals of the New York Academy of Sciences, Vol. 53, Art. 1. ROY WALDO MINER, Editor. Published by the Academy, New York, 1950. 219 pp. 15.6 x 23.5 cm.

This publication of the New York Academy of Sciences constitutes a series of papers presented during a conference on the Mechanism and Evaluation of Antiseptics held by the Section of Biology of the New York Academy of Sciences, October 28 and 29, 1949. Publication of the entire series of papers was made possible by grants-in-aid from a number of pharmaceutical manufacturers. Thus reviews relating to the mechanism and evaluation of antiseptics have been brought together in one place. In considering the possible mechanism of antiseptic action, "at least three papers in this monograph report that antiseptics seem to cause bacteria to lose proteins and electrolytes to the surrounding medium, and these suggest a profound alteration in the permeability of the membrane surrounding the organism. Such effects in either direction (increase or decrease of permeability) would prevent the organism from maintaining equilibrium with its environment and would constitute a sufficient mechanism of death."

The scope of the monograph can best be illustrated by reproducing the titles of the papers presented during the Symposium and the names of the authors of each.

The Metabolic Action of Streptomycin. By W. W. Umbreit

The Effect of Penicillin upon Protein Synthesis by Bacteria. By Rollin D. Hotchkiss

The Colloidal Nature of Antibiotics. By Ernst A. Hauser

New Antibiotics: Factors Influencing Their Antimicrobial Activity. By Gladys L. Hobby, Tulita F. Lenert, and Nancy Dougherty

The Effect of Surface-Active Agents on Antibiotics: An Informal Report. By Eleanor A. Bliss and Patricia T. Warth

Studies on the Synergism Between Bacitracin and Penicillin: Correlation of *In Vivo* and *In Vitro* Results. By Balbina A. Johnson and Frank L. Meleney

An Approach to Correlation between *In Vivo* and *In Vitro* Activities of Antitubercular Compounds. By Richard Donovick, Geoffrey Rake, and E. O. Titus

Evaluation of the Paper Disc Assay Procedure and Its Correlation with Phenol Coefficients. By Robert K. Hoffman and Charles R. Phillips

Mechanism of Action and Neutralizing Agents for Surface-Active Materials upon Microorganisms. By C. A. Lawrence

The Meaning of Bacteriostasis, Bactericidal Effect, and Rate of Disinfection. By Philip B. Price

The Interaction of Surface-Active Agents and Proteins. By Harold N. Glassman

Conductometric Studies of Bactericidal Mechanisms. By D. N. Eggenberger, L. A. Harriman, M. J. McClory, D. Noel, and H. J. Harwood

A New and Direct Approach to the Evaluation of the Germidical Efficiency of Semisolid Pharmaceuticals. By Milton J. Foter and Lester L. Nisonger

The Role of Antagonisms in the Evaluation of Antiseptics. By Emil G. Klarmann

Antiseptics under the Food, Drug, and Cosmetic Act. By Glenn G. Slocum

Present Trends in Methods of Testing Antiseptics. By G. F. Reddish

Halogens and Their Mode of Action. By Henry C. Marks and Frede B. Strandskov

Iodine as an Antiseptic. By Louis Gershenfeld and Bernard Witlin

Bacterial Resistance. By Orville Wyss

The Relationship of Concentration and Germicidal Efficiency of Ethyl Alcohol. By Harry E. Morton

Evaluation of Mercurial Compounds as Antiseptics. By Frank B. Engley, Jr.

Assay of Antiseptics at Different Times after Application to Human Skin. By H. M. Powell and C. G. Culbertson

Mercurials as Antiseptics. By John H. Brewer

Dr. Herbert L. Davis, of Ethicon Suture Laboratories, New Brunswick, N. J., deserves praise for having instigated the program leading to the Conference. He and the New York Academy of Sciences have performed a useful service of general interest to bacteriologists, but of especial interest to bacteriologists involved in the testing of antiseptics in pharmaceutical manufacturing laboratories and in those of law enforcement agencies.

Textbook of Organic Chemistry. 3rd ed. By E. WERTHEIM. The Blakiston Company, Philadelphia, 1951. xii + 958 pp. Illustrated. 16 x 23.5 cm. Price $5.

This book is a comprehensive and interesting presentation of the subject of organic chemistry for the college student. Under proper guidance and limitation, it can serve as a text for pharmacy students and for chemistry-major students. The clarity of presentation, combined with a fine index, makes it especially useful as a refresher reference.

The questions and bibliography at the end of each chapter add to the value of the text. The style, arrangement, format, and type of the book are excellent. The theoretical concepts of organic chemistry and the factual material are developed and presented clearly and concisely, and in a manner designed to attract the attention of the student and stimulate his interest in further reading on the subject in the many excellent references cited at the end of the chapter.

The author has drawn freely from tabulated data to illustrate the text. Pictures of outstanding chemists, with a brief sketch of the men and their important contributions, are included. An appendix includes a glossary of well-chosen terms, a chronological table of the developments in organic chemistry, a table of organic radicals, a table of physical data of many important organic chemicals, and other information.

The thoroughness of the book is indicated right at the beginning by quotations pertaining to science, truth, and knowledge, not by one or two, but by eleven eminent authorities. The usefulness and acceptance of the author's work is indicated by this third edition, which includes references up to January 1, 1951.

Scoville's The Art of Compounding. 8th ed. By GLENN L. JENKINS, DON E. FRANCKE, EDWARD A. BRECHT, and GLEN J. SPERANDIO. The Blakiston Company, Philadelphia, 1951. vii + 515 pp. Illustrated. 18.5 x 26 cm. Price $7.50.

This is a fine book for the student and the practitioner of pharmacy. The authors have performed their work of revision in the splendid manner that has marked the work of the earlier revisers of this perennial leader in the field of pharmaceutical education.

The pharmacist who is called upon to perform some compounding task after a lapse of many years will find a brief refresher course presented here as concisely as possible. The teacher of pharmacy will find this book especially useful because of its selections of prescriptions illustrating the different types of preparations and the many prescriptions for criticism and practice.

The book has so many good points that its few limitations cannot detract from its value. The increasing awareness of the need to stress precision in the compounding of prescriptions is shared by the authors, but they have apparently sacrificed stronger appeals to the student, perhaps to keep the book from becoming any heavier. Only a torsion-type balance is illustrated, and no stress is placed upon the importance of testing the prescription balance periodically. Among the prescription bottles illustrated is a graduated prescrip-

tion bottle. Even the manufacturers of prescription bottles state that these graduations are not reliable, especially those indicating smaller volumes. Some states have passed laws banning the use of graduated prescription bottles.

The format and type are excellent for easy reading. But it would be difficult to follow the advice given for shaving theobroma oil by holding the oil "in a piece of powdered paper to prevent excessive melting...."

The chapters on incompatibilities are exceptionally well presented. The nomenclature and vocabulary review, the discussions of isotonic and buffered solutions, the new material on solutions for special purposes and allergenic solutions, and the list of frequently prescribed proprietary and trade name medicinals are effective aids for the practicing pharmacist.

Annotated Bibliography of Vitamin E, 1940–1950. Compiled by PHILIP L. HARRIS and WILMA KUJAWSKI. The National Vitamin Foundation, Inc., 150 Broadway, New York, 1950. 184 pp. 21.3 x 27.3 cm. Price $3.

The need for this *Annotated Bibliography of Vitamin E* is appreciated when it is recalled that during the ten-year period between 1940 and 1950, more than 1,500 scientific papers relating to vitamin E were published. In order to aid physicians, biologists, chemists, and pharmacists in keeping abreast of new developments in vitamin E research, this annotated bibliography has been prepared.

The brief abstracts accompanying most of the references emphasize the physiological more than the chemical or medical aspects. The principal results recorded are summarized in a factual manner, and no attempt has been made to evaluate or criticize the work recorded. Only references to scientific literature appearing between 1940 and 1950 have been included. A bibliography of literature relating to vitamin E published prior to 1940 has been made available in a publication similar to the current one which was prepared several years ago by Merck & Co., Inc. The authors of the most recent *Annotated Bibliography of Vitamin E* are Philip L. Harris, Head of the Biochemical Research Department, and Wilma Kujawski, Librarian, of the Distillation Products Industries, of Rochester, New York.

The book is highly recommended to anyone interested in obtaining information relating to vitamin E.

The Biochemistry of Inositol. By EDWARD R. WEIDLEIN, Jr. Bibliographic Series Bulletin No. 6. Mellon Institute, Pittsburgh, Pa., 1951. v + 53 pp. 15.5 x 23 cm. Price: free.

During recent years inositol has been classified by some authorities as a vitamin belonging in the B complex group although no human requirement for it has yet been established. A lipotropic action in humans under certain conditions has been observed. This and other evidence resulting from animal studies accumulated mainly during the past ten years indicates that inositol may possess important nutritional and biological activities. Although the term "inositol" is used in a generic sense to include the nine different isomers of hexahydrocyclohexane, the name is applied in a specific sense to the *meso-*, or iso-, form. It is this form that has been known for more than 100 years, which is widely distributed in nature, and which may find use as a drug for the treatment of certain pathological conditions such as chronic hepatitis and cirrhosis in man.

The appearance of this booklet is timely because it presents a readily available comprehensive review of all the literature relating to inositol. The author has summarized existing information in an interesting style designed to serve as a basis for a critical evaluation of the relationships of inositol to other recognized factors in nutrition and biology. The scope can be visualized best by noting the titles of the chapters which are as follows:

I. Definition, Structure, Isomers and Derivatives of Inositol
II. Biochemical and Chemical Synthesis Inositol
III. Methods of Determining Inositol
IV. The Occurrence of Inositol
V. Inositol as a Microbiological Growth Factor
VI. Inositol as a Factor in Animal and Human Nutrition
VII. The Lipotropic Action of Inositol
VIII. Relationships between Inositol and Cholesterol
IX. Other Physiological Effects of Inositol
X. Inhibitory Analogs of Inositol
XI. Literature Cited

In the final chapter, 324 literature references are cited. The author and his sponsors have made available information that should prove of especial value to research specialists and others interested in existing knowledge concerning inositol.

Medicinal Chemistry. Vol. I. C. M. SUTER, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1951. vii + 473 pp. 15.5 x 23.5 cm. Price $12.

This book provides comprehensive and systematic summaries of available data on the biological properties of substances in the fields that have been studied. Each chapter includes references to the compounds that have been tested for a particular type of pharmacological activity. The data are presented largely in tabular form, according to chemical groups or series. Discussions of the relationships between chemical structure and pharmacological activity are included, wherever possible, in the concise reviews presented at the beginning of each division of the material. It appears that where less definite data are available, the review and discussion tend to lengthen. Under "Antibiotics from Plants," 50 pages of interesting material fortified with 277 references are followed by 8 pages of tabulated results of screening data on plants showing antibacterial properties. Under "Antithyroid Compounds," 33 pages are devoted to review and discussion, followed by tabulated data occupying 117 pages. Each chapter of this volume contains brief discussions of methods of synthesis and pharmacological test procedures.

The great value of this volume to those who are engaged in or who will enter upon investigative studies of synthetic medicinal chemistry rests mainly upon the many hours of searching, correlating, and tabulating that can be saved. This work has been

done by men who are competent in each division of medicinal chemistry that has been included in this volume.

The material included in this volume is presented under the following headings: 1. Antithyroid Compounds; 2. Antispasmodics: Derivatives of Carboxylic Acids; 3. Antibiotics from Plants; 4. Benzoates and Substituted Benzoates as Local Anesthetics; Analgesics: A. Aralkylamines; Analgesics: B. Partial Structures Related to Morphine. An index is included.

This book is not intended to serve as a textbook on the chemistry of medicinal products, but it is a reference work so valuable that the demand for it should be limited only by the number of individuals interested in this field of work and study.

Tablet Coating. By RONALD CLARKSON. Drug and Cosmetic Industry, New York, 1951. 75 pp. Illustrated. 14 x 21 cm. Price $4.

This little book contains valuable information in its limited field. It clearly presents the important facts with the minimum use of words. The author states that coating consists of a series of basic operations, each of which is relatively simple, yet each one must be carried out to perfection in order for the following operation to be successful. The most expedient method of learning to coat is by actually working under the watchful eye of an experienced coater; and the only reason for this book is that there are not enough places conveniently located to instruct all those desiring this knowledge.

The reasons for coating tablets are given. The apparatus required, and the steps and formulations required for subcoating, smoothing, coloring and finishing, and polishing, as well as white coating and enteric coating, are discussed. Procedures for reworking spoiled tablets are given, and erudite tips to the student of the art are included.

Fluoridation in the Prevention of Dental Caries. Council on Dental Health, American Dental Association, Chicago, 1951. 28 pp. 17.2 x 24.2 cm.

This booklet has been prepared by the American Dental Association to provide general information for the guidance of dental, medical, and public health officials in recommending fluoridation to city officials.

The fluoridation of public water supplies is generally accepted as one of the means whereby the prevalence of dental caries may be reduced. It possesses the advantage of easy application for large numbers of people and is relatively low in cost. Moreover, the effectiveness of fluoridation is greater than other preventive methods such as toothbrushing, restriction in the consumption of concentrated sugar, and topical application of sodium fluoride, because it reaches all users of public water supplies.

The booklet is recommended to those requiring general information on the subject of fluoridation, but detailed technical information must be obtained from other sources such as the State Dental Director or the Sanitary Engineer of the Health Department.

BOOKS RECEIVED

New Products and Services. Arthur D. Little, Inc., 30 Memorial Drive, Cambridge 42, Mass., 1951. 11 pp. 22 x 28 cm. Price: free.

Angewandte Pharmazie. 3rd ed. By WALTHER KERN. Wissenschaftliche Verlagsgesallschaft m.b.H. Stuttgart, Germany, 1951. xii + 454 pp. Illustrated. 17 x 24 cm. Price Lw. 38.

Mechanism and Evaluation of Antiseptics. Annals of the New York Academy of Sciences, Vol. 53 Art. 1. ROY WALDO MINER, Editor. Published by the Academy, New York, 1950. 219 pp. 15.6 x 23.5 cm.

Marine Products of Commerce. 2nd ed. By DONALD K. TRESSLER and JAMES MCW. LEMON. Reinhold Publishing Corporation, New York, 1951. xiii + 782 pp. Illustrated. 16 x 23.8 cm. Price $18.

Pharmaceutical Botany. 7th ed. By HEBER W. YOUNGKEN. The Blakiston Company, Philadelphia, 1951. xviii + 752 pp. Illustrated. 16 x 23.5 cm. Price $7.

History of Pharmacy. 2nd ed. By EDWARD KREMERS and GEORGE URDANG. J. B. Lippincott Company, Philadelphia, 1951. xiv + 622 pp Illustrated. 15.5 x 23.5 cm. Price $7.50.

Medicinal Chemistry. Vol. I. By ALFRED BURGER. Interscience Publishers, Inc., New York, 1951. xviii + 577 pp. 16 x 23.5 cm. Price $10.

Pharmazeutisches Taschenbuch. 4th ed. By HANS KAISER. Wissenschaftliche Verlagsgesellschaft m.b.H., Stuttgart, Germany, 1951. viii + 785 pp.. 13 x 17 cm. Price DM 28.

Annotated Bibliography of Vitamin E, 1940–1950. Compiled by PHILIP L. HARRIS and WILMA KUJAWSKI. The National Vitamin Foundation, Inc., 150 Broadway, New York, 1950. 184 pp. 21.3 x 27.3 cm. Price $3.

The Enzymes—Chemistry and Mechanism of Action. Vol. I, Part 2. Edited by JAMES B. SUMNER and KARL MYRBACK. Academic Press, Inc., New York, 1951. x + 637 pp. 16 x 23.5 cm. Price $12.80.

Pharmacognosy. By ROBERTSON PRATT and HEBER W. YOUNGKEN, JR. J. B. Lippincott Company, Philadelphia, 1951. xi + 644 pp. Illustrated. 15.5 x 23.5 cm. Price $8.50.

Kurze Einfuhrung in die Galenische Pharmazie. 2nd ed. By HANS WOJAHN. Verlag von Theodor Steinkopff, Dresden, Germany, 1951. 216 pp. Illustrated. 16 x 23.5 cm.

To you and us:

"How good" is more important

than "how many"

It's to your advantage when we concentrate on fewer fine chemicals.

One of our specialties:

bromides

AMMONIUM • SODIUM • POTASSIUM

This company is *known* for *certain* pharmaceutical chemicals—less than a hundred in all.

When you order any one of these you can be sure it's "good." We've concentrated on *fewer* fine chemicals and we make them to predetermined standards of purity and uniformity—standards beyond those generally expected.

Let's consider Baker's Bromides. Experience and skill have made possible Bromides in the proper range of particle size to permit direct compression into tablet form without additional grinding operations.

At the same time the small particle size and extreme freedom from cloudiness provide quick, clear solubility in liquid preparations.

With today's need for watching costs while keeping quality high, you need fine chemicals that do several jobs well.

When you buy fine chemicals, consider the "extras" that Baker's concentration on fewer fine chemicals makes possible. Specify Baker's! Address: *Fine Chemical Division*, J. T. Baker Chemical Co., Phillipsburg, N. J.

Baker CHEMICAL

Baker Chemicals

REAGENT • FINE • INDUSTRIAL

Eliminate Costly Product Contamination With PYREX Brand Bottles

Sensitive and expensive pharmaceuticals and antibiotics can easily be damaged in preparation, storage or shipment by improper containers. You can protect yourself against such losses by standardizing on PYREX Bottles.

The chemical stability of PYREX Bottles assures maximum protection against pH change. In addition, the glass from which these PYREX brand glass No. 7740 bottles are made, has a very low coefficient of expansion. This property makes possible repeated heat sterilization with a minimum of breakage, and at the same time permits heavy wall construction to give high mechanical strength, thus assuring long economical service life.

PYREX Bottles are available in standard sizes ranging from 60 ml to 12 gallons. For further information on standard or special bottles, write to Corning Glass Works. We are glad to be of service.

CORNING GLASS WORKS, CORNING, N. Y.

Corning means research in Glass

1851 • 100 Years of Making Glass Better and More Useful • 1951